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Georgia State Univ, Atlanta

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Workshop

Microbial Degradation of Pollutants in Marine Environments

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PROCEEDINGS OF THE WORKSHOP: MICROBIAL DEGRADATION OF POLLUTANTS IN MARINE ENVIRONMENTS

Pensacola Beach, Florida
9-14 April 1978

Edited by
A. W. Bourquin and P. H. Pritchard
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ENVIRONMENTAL RESEARCH LABORATORY
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FOREWORD

As man continues to develop and utilize new organic chemicals, the potential for environmental pollution increases accordingly. Once a chemical enters the environment, its eventual transfer to the aquatic environment becomes a distinct possibility. Increasing evidence indicates that a thorough knowledge of the fate of these chemicals is required. Inherent in any fate study is the need for an accurate assessment of the potential for the microbial degradation of the pollutant. However, information on biodegradation, specifically in aquatic environments, is sparse. Data from laboratory and field studies with soil microorganisms are often based on assumptions that have no sound scientific basis or consensus, regardless of the fact that aquatic environments appear sufficiently unique to demand separate consideration.

The microbiologist has no consistent guideline regarding appropriate techniques for the determination of recalcitrance in aquatic environments. Further, microbiologists in general have not provided the chemical industry and regulatory agencies with evaluations of chemical structures that can or cannot be degraded by aquatic microbial populations.

The Workshop on Microbial Degradation of Pollutants in Marine Environments was organized to offer representatives from government, academia, and industry a forum to examine key issues regarding the future direction and focus of scientific investigations in this field. Participants addressed a number of questions of primary concern: What are the differences and similarities between biodegradation processes in aquatic, terrestrial, and laboratory environments? Should a separate research effort determine biodegradation potential in aquatic environments or can adequate information be obtained from other sources? What methodological criteria must be established to provide interchangeable degradation information? What constitutes a recalcitrant molecule? What is the potential for a particular environment or its laboratory simulation to naturally dispose of a polluting chemical? We hope that this publication of the workshop proceedings will provide an up-to-date reference for professionals involved in the fate, regulation, and production of potential aquatic pollutants.

Thomas W. Duke

Thomas W. Duke
Director
Environmental Research Laboratory
Gulf Breeze, Florida

ABSTRACT

This international workshop, held April 10-14, 1978, at Pensacola Beach, Florida, focuses on pertinent issues related to the scientific investigation of microbial degradation of organic chemicals in aquatic environments. Participants discuss methodological criteria for these investigations and the need for biodegradation studies. Speakers and contributed papers for open sessions explore these topics: (1) biochemistry of microbial degradation; (2) transformation in aquatic environments; (3) compartmentalization in aquatic environments; (4) biodegradation in microcosms; (5) degradation methodology; and (6) persistence and extrapolation. Discussions within each session are presented. These proceedings conclude with a summary report and workshop consensus reports drafted by special task groups with recommendations concerning the research, production, and regulation of potential aquatic pollutants. This report is submitted in fulfillment of Contract No. 68-03-1325 by Georgia State University under the sponsorship of the U.S. Environmental Protection Agency. Work was completed as of 30 December 1978.

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We wish to express our appreciation for the tremendous help of the editorial committee in reviewing and editing the final report of this workshop, and of the advisory committee in establishing workshop goals and reviewing abstracts of contributed papers. We also are grateful for the encouraging remarks and cooperation of the participants and session chairmen. Ms. Peggy Wells, our workshop secretary, deserves special recognition for her efficient and gracious handling of all aspects of the workshop from meager tasks to the coordination of both professional sessions and social events.

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INTRODUCTION

THE ULTIMATE SINK

KEYNOTE ADDRESS

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ABSTRACT

The discussion of the marine environment as a site for degradation processes is introduced by reviewing some fundamental concepts of microbial ecology. The effective environment for bacterial and fungal activities is the micro-habitat of the individual cell. Characteristics of the aquatic environment are closely related to the physical and chemical properties of water in general, and of seawater in particular. On the macro-scale, the marine environment is divided, more for logistic than for microbiological reasons, into estuaries, littoral zones, water column, sediment, etc. For open ocean waters, the extreme dilution of metabolizable organic materials and pollutants is characteristic, and poses specific problems of microbial uptake and degradation. As an example, a microbial degradation study in the deep sea is discussed with emphasis on hydrostatic pressure and low temperatures as environmental factors. Microbial activities were reduced at deep sea conditions, and barophilic responses of natural microbial populations were not found. The consequences for offshore and deep sea dumping of organic waste materials are discussed.

INTRODUCTION

The ocean had to endure this not so romantic slogan, the "ultimate sink," ever since decomposition processes of organic and inorganic materials have been considered on the global scale. Slogans, however, are at least as often wrong as they appear to be downright. Inorganic marine deposits and solute complexes indeed represent accumulative evidence of global geological processes, but does this concept hold in the biological realm? In setting the stage for this workshop on Microbial Degradation of Pollutants in Marine Environments, it appears useful to attempt an answer to this question and to discuss briefly the environmental characteristics of marine microbial habitats

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with respect to potential degradation processes.

As a general principle, organic materials are produced as well as degraded by metabolic processes of organisms. If this recycling was complete in a given time period, and if the above principle was not riddled with exceptions, the term "ultimate sink" for biodegradation makes little sense for any particular environment. Considering, however, that a substantial number of organic materials are, or become during breakdown, recalcitrant to further biodegradation (a topic discussed in detail by Alexander [1]), and that more and more man-made and non-metabolizable products become part of our environment, many of these compounds will ultimately reach the ocean. Above all, however, it is the mere size of the marine portion of the bisophere which constitutes its significance as a site of biodegradation. Considering the average depth of the world oceans at 3800 m, as compared to 2 or 3 orders of magnitude less for the terrestrial and freshwater environment, more than 99% of the biosphere consists of seawater (13). Certainly, a large portion of this immense volume compares to a marine desert, while some small fractions, estuaries and littoral zones, are similar in their biological turnover rates to highly productive terrestrial and freshwater environments.

THE MICRO-HABITAT

Before we look into the characteristics of the various marine environments on the macro-scale, however, we have to remind ourselves that the typical habitat of any individual organism has to be considered in the perspective of its own dimension. The vegetative microbial cell with its relatively large and metabolically reactive surface responds quickly to types and concentrations of utilizable substrates and to changes of physico-chemical conditions of its immediate surroundings. As a consequence, "the effective habitat of a microorganism is its *micro*-habitat, the immediate surrounding of the cell in a compatible scale of space and time in relation to the radius of its metabolic action and interaction" (4). As a corollary, it is hazardous to describe microbial isolates by macro-environmental characteristics, e.g., estuarine or deep sea bacteria, before typically adaptive traits have actually been found. We know too well the ubiquity of microorganisms and their ability to survive unfavorable conditions for a long time in resistant, non-vegetative stages. In addition, most of our means to look at the size and composition of natural microbial populations are indirect and selective.

A classical case is that of Nathanson's (12) abortive attempt, in the wake of systematic monograph-writing on marine plants and animals in the late 1800s, to describe the bacterial flora of the Gulf of Naples. After years of hard work he gave up when he realized that the number of isolates kept increasing without yielding the slightest clue whether they were typical for this particular environment, the Gulf of Naples. His vain attempt has been repeated many times since with respect to marine environments of various descriptions.

More recently, the predominant significance of micro-habitats succeeded in eliminating the sharp dividing line between aquatic, terrestrial and even medical microbiology. In ecological research the distinction between these academic disciplines is now more and more de-emphasized under the label of basic and applied environmental or biogeochemical microbiology.

THE MARINE ENVIRONMENT

There are, of course, certain microorganisms specifically adapted to live in aquatic habitats. Characteristically, many of them are photo- or chemolithotrophic, or have certain morphological traits, such as being sheathed, stalked or prostecate forms. Although these organisms will be indirectly involved in biodegradation processes, our primary concern has to be with those heterotrophic bacteria which play the main role in the breakdown of pollutants of non-marine origin. Such bacteria are usually highly substrate-specific, but they themselves may or may not be of marine origin. For example, the decomposition of phenolic compounds in seawater may be determined a) by the specific activity of a marine pseudomonad or b) by the constraints of the marine environmental conditions on the metabolism of a non-marine pseudomonad. In many cases, we will not be able to separate between a) and b).

What, then, are the basic properties that characterize the aquatic, and specifically the marine, in contrast to the terrestrial environment? The basic feature of the predominantly aqueous environment, in contrast to more or less dry soils, "are the physico-chemical properties of water, especially its ability to carry those organic and inorganic chemical species which are essential and readily available for microbial metabolism in dissolved and ionic form. The mobility of nutrients in water by diffusion and currents produces the characteristic constancy of environmental conditions upon which many of the typically aquatic microorganisms depend" (4). In principle, the only additional and specific characteristics of marine habitats appear to be the salt complex of seawater (more its constancy than its particular composition) and hydrostatic pressure in deeper waters. The lingering doubt in the existence of truly marine bacteria, not satisfied by growth response on seawater agar or obligate sodium requirement, is only presently being more and more removed by accumulating evidence in Baumann's systematic assessment of marine heterotrophic bacteria (e.g., Baumann et al. [2, 3]). Although many bacteria of non-marine origin grow well in seawater, the majority of the marine isolates clearly exhibit a number of well defined metabolic differences. At this stage it is too early to speculate on whether and how these differences have an effect on the characteristic patterns of biodegradation in marine versus terrestrial environments.

After having pointed out the significance of the *micro*-environment in microbial ecology, any subdivision of the marine environment on the macro-scale may appear to be of little use. However, there are a number of practical reasons for doing so. The mere logistics of field work is one. Another is the technical differences in working with rich, shallow water sediments or oligotrophic pelagic seawater, etc. Summarizing from the literature, the following six marine habitats have been designated as such and individually studied: Esturaires; Littoral Zones (beaches, reefs); Shelf: water column and sediment; Open Ocean: water column and sediment.

Possibilities and needs for further subdivisions are obvious. Of special microbiological interest are interfaces as habitats. The water/air interface has physical and chemical properties which give rise to the highly concentrated "neuston" populations. They are important in the inoculation and degradation of oil slicks. Solid/water interfaces, recently treated comprehensively by Marshall (11), provide the habitat for "Aufwuchs" populations. Microbial growth takes place not only on the surface of utilizable organic substrates but also on inert suspended particulate matter, a topic summarized by Jannasch and Pritchard (6). Interfaces between soluble materials are of similar importance as demonstrated by the rich microbial populations surrounding oxic/anoxic layers of waters in estuaries and other highly productive marine environments. With the exception of some special areas as the well studied Black Sea and Cariaco Trench, where stagnant anoxic water has accumulated, offshore waters and deep sediments are rarely found to be anoxic largely due to the comparatively low rates of oxygen uptake.

MICROBIAL DEGRADATION IN THE DEEP SEA, AN EXAMPLE

At this point, let me diverge a bit from my proper duty to introduce this workshop by discussing general concepts, and let me use some of my own recent work as an example of specific marine-oriented research on microbial degradation. Also, I believe, no one else at this workshop is dealing with the deep sea as a special marine environment. At the beginning of this talk, I mentioned that 99% of the biosphere by volume comprises marine environments. If we go beyond the 200 m depth of the shelf and let the deep sea deliberately begin at a depth of 1000 m, we come to the surprising result that still 75% of the biosphere is located below that line; in other words, it is subjected to 100 or more atm of pressure, temperatures in the range of 1-4°C and, of course, receives no light.

It is important to realize that most of this huge area is far removed from direct inputs of organic sources of energy. Its character is oligotrophic, the deeper the more so. Most of the organic matter produced photosynthetically in surface water is decomposed in the upper few hundred meters. The huge volume of deep sea water appears to make the mere dilution of pollutants almost indefinite. At the same time, as we found in earlier chemostat work, the microbial degradation of substrates is slowed down significantly if they are diluted beyond a critical point (5). Thus, in spite of the large volume of water, certain pollutants have been found everywhere in the ocean. It is self-evident that metabolic processes in higher organisms as well as characteristic chemical reactions of seawater and sediments participate in transformations and breakdown of pollutants.

Our interest in the deep sea has been intensified by the fact that 50 million tons of solid waste materials are dumped into the sea annually by this country alone. Since shelf waters comprise over 90% of the marine fisheries, there is a tendency to use deeper water as dumping sites for materials that might interfere with bottom trawling. Over decades of work on benthic deep sea populations and from many recent experimental studies with research submersibles, the general impression is that of a highly specialized and fragile community. Our question was, how will these populations be affected by the input of extragenous materials in unusual quantities? Will microbial enrichment occur in a similar way it does in estuaries or shallow waters? In short, can the deep sea be considered as an active treatment system for organic waste materials?

Over the last seven years, we have deposited and recovered a large variety of organic substrates in depths from 1800 to 5300 m. These in-situ incubation studies eliminated the potential effect of decompression which occurs during the recovery of deep sea samples for the subsequent incubation in pressurized laboratory cylinders. In some of these field experiments, media-containing bottles were inoculated with the aid of the research submersible *Alvin* directly on the deep sea floor, thus excluding all surface-borne microorganisms. This way we hoped to find enrichments of truly barophilic bacteria, which are defined as metabolizing at an increased rate at pressures higher than one atmosphere. Such a proposed indigenous microflora might be disproportionally outnumbered if surface-originated organisms were not excluded from the experiment. The substrates used in these quantitative studies included a number of organic acids, amino acids, carbohydrates (sugars and polysaccharides) and some complex materials such as starch, gelatin and agar. Semi-quantitative recoveries were made with samples of wood, refined cellulose, chitin, food stuffs and seaweed. Incubation times ranged from several days to 15 months. Since these studies aimed at the general question of bacterial metabolism under deep sea conditions, pollutants were not yet included. The specific techniques are discussed elsewhere (7, 8, 14).

As a general result, deep sea-adapted microbial activities were not found. The effect of increased pressure and low temperature appeared to be the reason for the slowdown of the measured activities as compared to laboratory controls incubated at equal temperature. This effect was the same in microbial populations exclusively collected at the deep sea floor and in those originated at the sea surface. In both cases decompression resulted in increased activity. In other words, no truly barophilic response could be recorded. These results were substantiated by growth and uptake studies using pressure-retaining samplers which permitted measurements on deep sea populations that were never subjected to changes of their indigenous pressure and temperature (9, 10).

It is very likely that these results will also apply to the microbial degradation of pollutants wherever they reach the deep sea. They also indicate that a substantial accumulation of certain materials might occur in deep waters. In short, the deep sea has definite limitations as a dumping site for organic wastes. Several observations made with the aid of baited cameras deposited on the deep sea floor led our attention to the role of higher organisms in the removal of organic waste materials. It will be difficult to separate these effects in the deep sea entirely from microbial activities since much of it appears to take place in the intestinal tracts of invertebrates and fishes. Our work in this area is continuing.

Where does this leave us with respect to the "ultimate sink"? If "sink" or "dump" refers to places where discarded materials do persist, at least partially, then the open ocean and the deep sea, as by far the largest portion of the biosphere with its relatively limited degradation potential, might indeed be considered an ultimate sink. Development of needed monitoring technology and smooth cooperation between producers of new potential pollutants, government agencies, and independent research laboratories are paramount requirements.

THE WORKSHOP

The program of this workshop is based on the present-day state of the art in our knowledge of microbial breakdown processes, modeling of systems, the types of organisms involved, techniques of monitoring and the variety of pollutants existing at this time. Oil, herbicides and pesticides, DDT and PCBs, domestic and industrial sewage, are those pollutants foremost on the public's mind. Their slow disappearance from the polluted environment is generally understood to be caused by biological and chemical activities. Bacteria, who generally suffer from their bad reputation as one of man's worst enemies, have gained considerable favor by being mentioned in connection with environmental cleanups. To this day, the microbial ecologists, since Winogradski, are trying to put the image of microorganisms, tarnished by dominating medical viewpoints, in the right perspective. The present workshop, brought about ultimately by public interest, is a veritable success in these efforts.

The scene is set by the steering committee of this workshop to include a wide variety of topics in a number of well organized sessions. Let us discuss more than just deliver; conduct an actual workshop, not a mere convention. The outcome should be stimulating for the further development of research in microbial degradation, a field that will be of more and more importance as long as variety and quantity of pollutants in the environment are on the increase.

LITERATURE CITED

1. Alexander, M. 1973. Non-biodegradable and other recalcitrant molecules. Biotech. Bioeng. 15:611-647.

2. Baumann, L., P. Baumann, M. Mandel, and R. D. Allen. 1972. Taxonomy of aerobic marine eubacteria. J. Bacteriol. 110:402-429.

3. Baumann, P., and L. Baumann. 1977. Biology of the marine enterobacteria: genera *Beneckea* and *Photobacterium*. Ann. Rev. Microbiol. 31:39-61.

4. Jannasch, H. W. 1978. Microorganisms and their aquatic habitat. *In* W. B. Krumbein (ed.), Environmental biogeochemistry. Ann Arbor Sci. Publ., Ann Arbor, Mich.

5. Jannasch, H. W., and R. L. Mateles. 1974. Experimental bacterial ecology studied in continuous culture. Adv. Microb. Physiol. 11:165-212.

6. Jannasch, H. W., and P. H. Pritchard. 1972. The role of inert particulate matter in the activity of aquatic microorganisms. Mem. Ist. Ital. Idrobiol. 29 Suppl.:289-308.

7. Jannasch, H. W., and C. O. Wirsen. 1973. Deep sea microorganisms: in situ response to nutrient enrichment. Science 180:641-643.

8. Jannasch, H. W., and C. O. Wirsen. 1977. Microbial life in the deep sea. Sci. Amer. 236:42-52.

9. Jannasch, H. W., C. O. Wirsen, and C. L. Winget. 1973. A bacteriological, pressure-retaining, deep sea sampler and culture vessel. Deep-Sea Res. 20:661-664.

10. Jannasch, H. W., C. O. Wirsen, and C. D. Taylor. 1976. Undecompressed microbial populations from the deep sea. Appl. Environ. Microbiol. 32:360-367.

11. Marshall, K. C. 1976. Interfaces in microbiology ecology. Harvard University Press, Cambridge, Mass.

12. Nathanson, A. 1902. Unpublished correspondence with Anton Dohrn. Archives of the Zoological Station, Naples.

13. Sverdrup, H. W., M. W. Johnson, and R. H. Fleming. 1942. The oceans. Prentice Hall, Inc., Englewood Cliffs, N.J.

14. Wirsen, C. O., and H. W. Jannasch. 1976. The decomposition of solid organic materials in the deep sea. Environ. Sci. Technol. 10:880-887.

INTRODUCTION AND WELCOME TO THE WORKSHOP

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It is my pleasure to welcome you to Santa Rosa Island and to this Workshop on behalf of the U.S. Environmental Protection Agency and Georgia State University. As Director of the Environmental Research Laboratory, your host this week, I would like to invite each of you to tour the laboratory during your stay here. At the laboratory we study the impact of toxic organics on the marine environment. The primary emphasis in our research has been the effects of pesticides on marine organisms. Early in our history we concluded that in order to study effects of toxic organics, you certainly must have some knowledge of the fate of these materials. When we study fate of pollutants, we cover a variety of disciplines which play an active part in determining what happens to an organic compound when it enters the marine environment.

In the past we have dealt primarily with organochlorine compounds which were quite resistant to biodegradation. Therefore, fate research focused on other processes such as photolysis, hydrolysis and sorption kinetics. However, more recently our research program has become concerned with the "biodegradable" compounds such as organophosphorus and carbamate pesticides. This evolution has, of course, brought more emphasis to our studies on biological transformations. For this reason, we have scientists on our staff such as Al Bourquin, Hap Pritchard, and Dick Garnas who are involved in studies to determine biological and physio-chemical fate of organics in the marine environment. The importance of good scientific research in environmental studies is evident to all of us. Dr. Stern will be telling you about the pressing needs of the Office of Toxic Substances to develop good techniques to evaluate environmental fate information to make regulatory decisions. Even a cursory glance at the program shows we have some of the leading experts in the field present in this Workshop. We are extremely pleased to have this opportunity to host this Workshop and I want to second a remark of Dr. Jannasch in his keynote address. That is, the success of this meeting will be that it is not just one of lecturing and responding but one of a true workshop. I leave you with this challenge that there will be good intensive discussions on fate processes so that we can reach the goals of the workshop. Your participation is needed to reach these goals.

NEED FOR BIODEGRADATION STUDIES BY THE EPA

Arthur Stern
Office of Toxic Substances
U.S. Environmental Protection Agency
Washington, DC

INTRODUCTION

In 1976, the Toxic Substances Control Act (TSCA) was passed by Congress. The Act created the Office of Toxic Subtances (OTS) and charged that office with the responsibility of developing procedures whereby all chemicals brought into commercial use, other than pesticides, foods, drugs and cosmetics, would be evaluated with regard to their effect on the public health and the environment. One of the requirements imposed on OTS by the Act was the need to develop test standards, protocols and/or guidelines for use by industry. These would be employed to generate the data which would be used by the agency to make risk assessments. Tacit is the understanding that only state-of-the-art technology be incorporated into test protocols. Another important point is that the Act stipulates that testing requirements be reviewed annually for adequacy, since new developments often lead to better, more relevant and cheaper techniques.

At the present time, it is our intent to spell out test protocols in some detail. This would expedite the risk assessment process considerably and should ultimately result in faster adjudication of applications. Furthermore, advance detailing of the criteria upon which assessments will be made by the agency will allow potential applicants to make preliminary judgments of their own. This does not mean that published testing requirements will be totally inflexible. It is hoped that enough flexibility will be incorporated into such requirements to take into consideration the large variation in chemical structures, properties, end-uses and distribution. In short, our objective is the development of the minimal number of cost-effective testing requirements the data from which should effectively provide answers essential in making risk assessments. To this end, our present thinking is to arrange tests in a series of tiers. The evaluation of data generated by tests carried out in each tier will lead to a decision as to whether or not the next higher tier of tests will be required. The lowest tier will consist of relatively inexpensive short-term tests, while subsequent tiers would be characterized by more extensive testing requirements. All of this represents our current thinking and is a somewhat idealized concept. Since we are only starting out in the rule-making process, it is quite possible some elements of this concept may prove to be impractical and have to be modified accordingly.

ENVIRONMENTAL TESTING

Environmental testing includes the areas of ecological effects and chemical fate. There are many problems that are associated with the development of ecological-effects testing which I will not dwell on since our purpose this week is to consider one aspect of chemical fate. However, I would like to mention a few of these problems before moving on to the subject of persistence testing. The environment consists of several compartments, many ecosystems within each compartment and diverse phyla, genera and species of plants and animals within given ecosystems. The question of whether testing of single species in a laboratory bears any relevance to what happens in the real world must be answered ultimately. While this correlation is far from clear, we have to start somewhere and judgment on such correlations has to be reserved until more information is acquired. It is our present feeling that microcosm and field studies will be eventually needed to validate predictions made on the basis of individual species testing. These problems also happen to be germane to the area of biodegradation.

CHEMICAL FATE

Chemical fate, which is a part of the environmental picture, really consists of the two separate, but intimately related, areas of chemical transport and persistence. Conceptually, it is impossible to differentiate these areas but, from the standpoint of developing testing requirements, we have found it convenient to attack each separately. When it comes to interpreting data derived from test protocols, both areas must certainly be evaluated in concert.

CHEMICAL TRANSPORT

Some generalized predictions with regard to chemical transport can be made on the basis of physical/chemical characteristics of test materials. In other words, some concept may be derived from an analysis of a chemical's key physical/chemical characteristics regarding the environmental compartments which may serve as its major deposition site or sites. Persistence data give us some indication of the expected residence time of a chemical after deposition. Finally, some idea of the exposure potential of a chemical pollutant (within a few orders of magnitude) can then be derived by integrating transport and persistence data with information acquired from responses to Section 8 of the Act, i.e., production volume, production distribution, etc.

This analysis has several important applications. First, as stated before, it provides a rough idea with regard to environmental exposure. This, in turn, can serve as a guide in determining the dosage levels to use in both health and environmental effects testing. Second, certain environmental compartments can be ruled out as primary deposition sites and, thus, the magnitude of ecological testing required can be reduced.

PERSISTENCE TESTING

As stated earlier, persistence data allow some predictions to be made with regard either to the duration of a chemical's residence after deposition, if its application is intermittent or occurs only once, or to its turnover rate if its application is continuous. Chemicals which are found to be innocuous at low dosages can, if persistent, accumulate to hazardous environmental levels over a period of time. Further, chemicals which are degraded cannot be considered exempt from further concern as potential hazards. There are quite a few examples of degradation products posing a greater threat than the parent compound from which they originated. Thus, persistence testing must include some provisions for the examination of degradation products of non-persistent chemicals.

BIODEGRADATION

There are three general mechanisms by which chemicals can be degraded. These are photochemical, chemical and biological. Since our major concern this week is the latter, let me point out some of the problems we face in developing test standards/protocols for biodegradation.

First of all, literature on biodegradation testing is rather diffuse. While it is true that a good deal of work has been published on the degradation of organic compounds, much of it has been directed at the determination of metabolic pathways by which certain types of chemically pure materials are transformed by pure cultures in a well defined medium. Other studies which had more applied objectives (such as the stabilization of organics) usually only dealt with a restricted number of chemical types. This doesn't imply that the techniques used were necessarily limited to these types of chemicals but it does mean that a good deal of validation work would have to be carried out to determine the chemical limits of method applicability. There have been methods described which show some promise of general utility, such as the river die-away test, but these have certain inherent deficiencies that make their adoption as a sole means of biodegradation testing impractical. It is quite likely that ultimately we will have to require the use of a battery of test methods to accommodate the spectrum of variables that influence the extent and rate of biodegradation.

I would now like to list those issues which are particularly important in devising meaningful tests for biodegradation and which I hope will be addressed by the speakers and workshops during the coming week:

- Mixed vs pure cultures
- Sources of mixed and/or pure cultures
- Laboratory vs natural media
- Adapted vs unadapted cultures (continuous vs single slug or intermittent doses)
- Aerobic vs anaerobic testing
- Concentration(s) of toxicant to use in tests, i.e., differential responses to low and high concentrations of many chemicals run the gamut from inhibition to stimulation of growth

- Role of cometabolites
- Synergistic, commensal, symbiotic and antagonistic relationships between species in a natural environment.

Some other problems which are shared by all methods of degradation can be summarized as follows:

- Correlation of tests on isolated reactions in the laboratory with multiple reactions in nature
- Design of experimental conditions to meet those most frequently encountered in nature (pH; temperature; protective/concentrating influence of particulates such as soil, dust particles and suspended matter in aquatic environments)
- Additive vs non-additive effects of multiple routes of degradation
- Definition of "significant degradation"
- Analysis and identification of "most important" degradation products
- Testing requirements for identified significant degradation products.

I would like to conclude with the hope that you will: address the issues that must be resolved, those I have mentioned and others that you are aware of; identify methodology in the area of biodegradation that is almost, but not quite, state-of-the-art; and identify short- and long-range method development programs whose successful completion could improve our current capabilities in this area.

MISSION OF THE WORKSHOP

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The remarks of Drs. Stern and Duke emphasize the timeliness of this Workshop. With the legislative passing of TOSCA, creation of the Office of Toxic Substances, the proposed pesticide registration guideline in the Office of Pesticide Programs, and the new effluent guidelines, the need for more emphasis on biodegradation studies and a better understanding and interpretation of biodegradation results becomes increasingly important. That is the mission of this Workshop, to develop guidelines for better understanding and interpretation of biodegradation studies in aquatic environments.

In order to make a regulatory decision on any chemical entering the environment, we need to know what will happen to that chemical when it enters a particular environment. What is the potential for biodegradation in a given environment? If it is low, will the compound persist and cause the environment harm? If not persistent, what are the possible degradation products? What is the toxicity of these metabolites to non-target organisms in relation to the parent compounds?

With the increased usage of the new era of pesticides, the organophosphates as opposed to the organochlorines, information on the biodegradation of these compounds is even more important. Currently, much information on the fate of a chemical in aquatic environments is determined by techniques developed for soil or terrestrial studies. For example, in the proposed pesticide registration guidelines, section on aquatic metabolism, I quote: "The preferred substrate is sediment, but use of flooded soils may be inadequate." If this proposed method is adopted, do we have all the needed information for such a substitution? Can we really use soils in place of sediment when studying aquatic metabolism? These are the type questions I hope this group will attack this week.

The aquatic environment is unique. It should be studied with the incorporation of certain characteristic factors. The aquatic environment is an *open* system, encompassing many time-independent processes. At the same time, it is a *dynamic* system with continuously changing inputs--pollutants or nutrients. These substances may be put into the system in pulses, such that one does not have a continuous system.

The aquatic environment is a highly dilute system and pollutants entering this environment are diluted out rapidly to low levels. The diluted environment of aquatic microorganisms makes their survival capacities or physiology different from other microbes surviving in high nutrient environments. Microorganisms are continuously exposed to very low nutrient concentrations; therefore, the microbes are probably capable of growth at extremely slow rates. In fact, it is quite probable that aquatic microorganisms may have an entirely different physiology from microorganisms living in a soil environment. On the other hand, pollutants and nutrients are sometimes concentrated in specific interfaces of the aquatic environment, such as surface slicks and sediments. These highly concentrated areas offer a special ecological niche in an otherwise dilute environment. This is an area of concern to us. What role do these special environments play in the overall metabolism of aquatic microorganisms? All these factors seem to dictate a specific methodology for aquatic studies.

The aquatic environment is characterized by water continuously passing over sediments, as opposed to a soil environment where the water to soil ratios are such that water, most of the time, soaks into the soil with only occasional runoff. This movement of water over sediment causes changes in sorption/desorption kinetics of pollutants into and from sediments. The occurrence of suspended particulat[illegible] a unique characteristic of the aquatic environment and may affect the [illegible]radation rate of chemicals. Biodegradation in the sediments is a relatively unexplored area in aquatic ecology, particularly the role of sediments to initiate degradation prior to the involvement of planktonic bacteria. As in soil systems, pollutants are irreversibly bound in aquatic sediments. We must yet determine if these bound residues are biologically recyclible or inert. These are all factors that should be considered when designing studies or attempting to design standard tests for regulatory actions to determine fate in the aquatic environment.

In addition to the physical characteristics of the aquatic environments, the chemical characteristics of higher salinities and higher pH added to the complexities of biodegradation studies in marine environments. How these factors affect biodegradation is of primary importance to us at ERL-GB. The fate of a chemical in aquatic environments is an interaction of all these various processes on the chemical. None of the current methodologies are applicable to assess all of these processes in the aquatic environment. How other degradative processes affect biodegradation is an important factor in assessing fate in the environment. For example, environmental parameters such as salinity, pH, and other physical factors may alter the microbial community or affect its innate biodegradation potential. In the design of laboratory systems to assess biodegradation potential, type of water, sediments, ratio of sediment to water volume are factors for consideration to properly design systems to simulate environmental conditions.

I hope some of the topics I have touched on here will help to orient the discussion on the differences and similarities in biodegradation studies in terrestrial and aquatic environments. We have scheduled six task groups which will meet outside the formal sessions to intensify discussion in the areas of: 1) biochemistry, 2) transformations in aquatic environments, 3) compartmentalization in aquatic environments, 4) microcosm development, 5) methodologies, and 6) extrapolation and persistence.

BIOCHEMISTRY OF MICROBIAL DEGRADATION

Chairperson, David T. Gibson

TRANSFORMATION OF XENOBIOTICS BY MICROBIAL ACTIVITY

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ABSTRACT

The fate of xenobiotic compounds in the environment represents an area of intense contemporary interest, since disappearance, persistence, or partial transformation of such a compound determines its usefulness or its potential hazardous effect. There may be biological, chemical and physical factors that influence the fate of a xenobiotic, but the least predictable transformation is usually caused by microorganisms.

Mechanisms of microbial intereference with xenobiotics include the use of a compound as the source of both carbon and energy, cometabolic transformation, conjugation and polymerization reactions, or the mere accumulation of a chemical within a microbe. It is difficult to predict which molecular change can be expected by microbes, since each group of organisms, even various strains of one genus, can alter a selected molecule differently. Consequently, for numerous xenobiotics tens of metabolites can be formed. However, a certain mode of biological transformation can be anticipated in a specific environment if metabolic reactions or pathways with various microorganisms are determined, their formed intermediates and products identified, and the biochemical mechanisms established. To illustrate this concept, a study performed with the insecticide carbaryl is presented in which the metabolic activity of various organisms was followed.

The introduction into the environment of chemicals, both naturally occurring and man-made xenobiotics, causes changes in the various ecosystems. These can be beneficial or harmful. For millions of years, nature adapted to various natural organic products, but only in the last 30 years have numerous man-made organic compounds been used in the environment. Therefore, it is a recent task to investigate how nature or the environment can cope with the

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newly synthesized chemicals. It is obvious that chemical and physical factors influence the fate of xenobiotics, but most of the degradative activities are initiated by microorganisms. Furthermore, it is merely academic whether microbes are infallible in transforming a chemical, but it is an urgent environmental problem to determine the time span of recalcitrance and the chemical transformations that take place. A long resistance time of a chemical can result in its accumulation to toxic levels for different organisms. Furthermore, microbial metabolic processes can create new chemicals with undesirable or harmful effects. Metabolic changes of xenobiotic compounds in soil, freshwater, estuarine or marine environments appear to be caused primarily by bacteria, actinomycetes and fungi. However, there are considerable differences in the composition of the microbial populations in the various ecosystems and, consequently, different transformation reactions can be expected.

If a xenobiotic compound is exposed to a microbial species there are several possibilities for its transformation or inactivation: (a) the compound can serve as a substrate for growth and energy; (b) xenobiotic compounds can undergo cometabolism, i.e., microbes transform it but cannot derive energy for growth from it; (c) a xenobiotic molecule or an intermediate of it can be conjugated or polymerized with naturally occurring compounds; and (d) the xenobiotic is incorporated and accumulates within an organism.

The complete microbial degradation of an organic molecule to its inorganic components is desirable if one is interested in avoiding the persistence of a potentially hazardous compound in the environment. If a xenobiotic can be used in such a way, it is metabolized and fragmented to compounds that can be channeled into known oxidative cycles; thus, the organism can derive all the necessary energy. The phenomenon of complete biodegradation can occur often in nature, and it can be anticipated that usually several microbial species participate in the metabolic transformations.

Cometabolism, the transformation of a chemical by a microbe without deriving energy to support its growth, has been observed and studied only in recent years. From an environmental viewpoint, the phenomenon of cometabolism of xenobiotics has to be considered carefully, since this reaction generally does not result in extensive degradation of a molecule. Consequently, it is difficult to foresee if such a product reduces or increases toxic effects in the environment.

Higher organisms use conjugation systems to convert xenobiotics into an excretable form. Such a mechanism is usually not of importance for microbial metabolism, but microbes possess enzymes which cause such reactions. Examples can be found in soil or sediments where oxidative coupling enzymes can combine xenobiotic compounds, such as halogenated phenols or anilines, with humus compounds.

In many instances it was shown that xenobiotic compounds are incorporated into microorganisms by an active or passive accumulation mechanism. Not only live bacterial cells but also autoclaved ones show a similar uptake. This appears to indicate that a natural metabolic factor is not always involved in the accumulation process. Since aquatic microorganisms and plankton in freshwater and marine environments are an important nutrient source for a broad spectrum of aquatic filter-feeding organisms, their accumulation of xenobiotic chemicals can constitute a hazardous link in the food chain to fish and higher vertebrates. Therefore, the findings of extensive biomagnification by these organisms has to provoke considerable concern.

In the study of drug metabolism, most compounds are metabolized in higher organisms by non-specific enzymes that catalyze relatively few general reactions. Similar findings have also been made for microorganisms. One can assume that the important enzymatic reactions taking place have been described, and, consequently, it appears possible to anticipate the biotransformation of a certain chemical molecule. Although the aforementioned is true, many transformation mechanisms exist, and the tremendous variation of organisms with different enzyme systems can result in formation of numerous different products. Even a similar kind of mechanism can produce slightly different chemical products which themselves may require different transformation reactions. Some products may be easily degradable while others may be quite resistant to biological attack.

From these general remarks it can be concluded that it is a very complex problem to establish the metabolic fate of a certain xenobiotic substance. The major metabolic pathways of a compound have to be elaborated, but one must always be aware that specific circumstances can cause a deviation from such a pathway. Under various conditions or in different ecosystems a chemical can be transformed differently and the resulting product can vary considerably. However, knowledge of enzymatic reactions in the metabolism of chemicals, or their identified intermediates, should contribute to understanding transformation possibilities. Essentially, the biochemical processes have been investigated in model systems using isolated microbial cultures or enzyme systems. This appears, with presently available techniques, to be the only feasible approach, and is a prerequisite for establishing and predicting the possibility of microbial xenobiotic transformations in a natural environment.

To illustrate this concept, a study with the insecticide carbaryl (1-naphthyl-N-methylcarbamate), in which the metabolic activity of various organisms was followed, was performed in our laboratory. Carbaryl was selected as a pesticide since it is the most used carbamate insecticide in the United States, being registered for use in controlling more than 160 different insect pests and on 85 different food and fiber crops. Carbaryl was first synthesized in 1953 and introduced as a commercial insecticide in 1958, but did not receive widespread attention until it was recommended as a safe substitute for DDT. Although carbaryl is a relatively simple organic molecule, its metabolism is very complex and there is still much to be learned about the biochemistry of this compound (9). Chemical degradation of carbaryl by hydrolysis occurs with relative ease (especially in alkaline media) and it was first assumed that the predominant metabolic pathway of carbaryl is hydrolysis of the carbamate ester linkage.

It was possible to isolate several microorganisms capable of hydrolyzing carbaryl to 1-naphthol (1). In these studies it became evident that 1-naphthol is a more toxic compound to microorganisms that the original pesticide. This observation was confirmed with other organisms and, therefore, we concentrated our later studies on the metabolic fate of 1-naphthol. The existence of a non-hydrolytic pathway was first found with mammals, plants and insects (8), but studies with the soil fungus *Gliocladium roseum* also showed the formation of 4-hydroxy-1-naphthyl- and 5-hydroxy-1-naphthyl-N-methylcarbamate (10). Hydroxylation of the side chain also occurred and yielded 1-naphthyl-N-hydroxy methylcarbamate. The ability to hydroxylate carbaryl at different positions occurred with several other soil fungi, but the products differed qualitatively as well as quantitatively with the various fungi (2). The metabolism of xenobiotic compounds is often initiated by hydroxylation, but these results show that it is difficult to predict which exact molecular change, in this case hydroxylation, can be expected by a specific microbe. Each group of organisms, even various strains of one genus, can alter a certain molecule differently. For instance, *Aspergillus terreus* caused the formation of 1-naphthyl-N-hydroxymethylcarbamate as the major metabolic product while *A. flavus* had a stronger tendency to hydroxylate carbaryl in the ring position (Table 1).

TABLE 1. SIDE-CHAIN- AND RING-HYDROXYLATION OF CARBARYL (METHYL^{14} C-LABELED) BY VARIOUS SOIL FUNGI

Fungus	1-Naphthyl N-hydroxy-methylcarbamate	4-Hydroxy-1-naphthyl methylcarbamate	5-Hydroxy-1-naphthyl methylcarbamate
Aspergillus flavus	4352*	126	228
Aspergillus fumigatus	0	0	0
Aspergillus terreus	4854	59	262
Gliocladium roseum	829	254	408
Mucor racemosus	134	1275	834
Mucor sp.	65	406	666
Penicillium sp.	266	3342	1003

*Amounts of radioactivity (d.p.m.) detected.

It is obvious to assume that the subsequent metabolic transformation of various hydroxylated products will differ considerably. Further microbial alterations of the ring-hydroxylated carbaryl was not followed, but we investigated the metabolic transformation of 1-naphthyl-N-hydroxymethyl carbamate by *Aspergillus terreus* (11). 1-Naphthyl-carbamate was established as the next intermediate and subsequently 1-naphthol was formed (Fig. 1).

As previously outlined, 1-naphthol appears to be the major intermediate in the degradation of carbaryl, and therefore we studied the bacterial and fungal metabolism of this compound. Several bacteria isolated from river water were capable of degrading 1-naphthol (3). After 60 h of incubation with 1-naphthol-1-^{14}C, it was possible to trap 44% as $C^{14}O_2$, and 22% was recovered in the bacteria (Table 2). The release of C^{14}-labeled CO_2 clearly indicated that complete biodegradation of the chemical had taken place. In addition, it was found that 15-20% of radioactivity from 1-naphthol remained in the growth

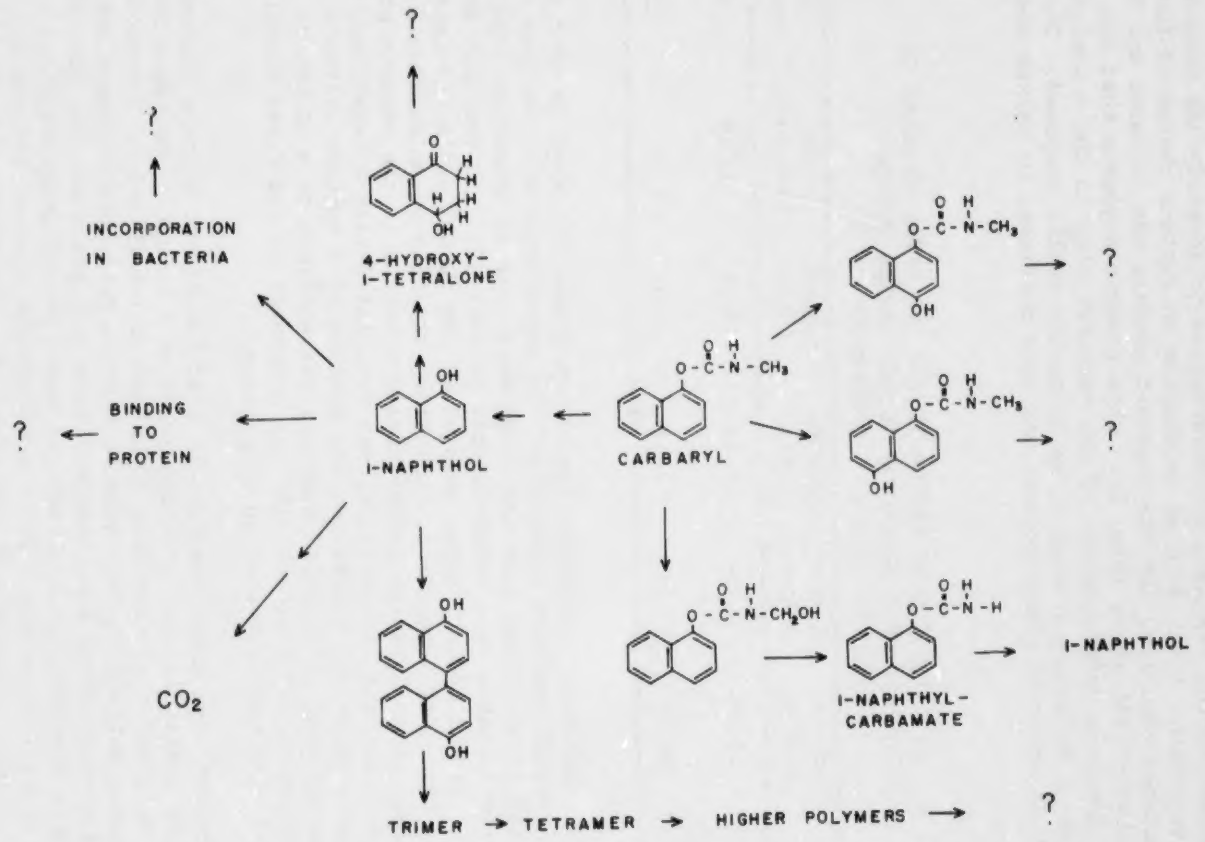

Figure 1. Metabolic transformation of the insecticide carbaryl (1-naphthyl-N-methylcarbamate) by various microorganisms.

medium, apparently without further change. This suggests that at least two different pathways are involved in degradation of 1-naphthol by the investigated bacteria. The radioactivity remaining in the growth medium was partitioned by ether extraction into an organic and an aqueous phase at a ratio of 35 to 65%, respectively. The ether extract was analyzed by thin-layer chromatography, but no attempt was made to characterize or identify the composition of the aqueous phase. After 48 h or incubation no further change of the formed metabolites occurred, and the dominant product was isolated and identified as 4-hydroxy-1-tetralone (Fig. 1). This product suggests that one alternate pathway involves hydroxylation of the naphthyl ring in the 4 position, and conversion of an aromatic ring to an aliphatic cyclic compound. The existence of such an apparent shunt pathway has been confirmed by Walker and coworkers (13).

TABLE 2. DISTRIBUTION OF RADIOACTIVITY (%) AFTER 60 HOURS OF INCUBATION OF A BACTERIUM IN THE GROWTH MEDIUM CONTAINING 20 ppm OF 1-NAPHTHOL-1-^{14}C

	Growth medium	Ether phase	Aqueous phase	Trapped $^{14}CO_2$	In bacteria	Total recovery
Bacterium	16.5%	5.5%	11.0%	43.5%	22.0%	82.0%
Non-inoculated control	100.0%	97.5%	3.0%	0.0%	--	100.5%

Knowledge related to transformation of 1-naphthol by fungi is even more sparse than bacterial naphthol metabolism. In experiments with various *Rhizoctonia* species it was found that the fungi were able to transform 1-naphthol from an ether-extractable to a water-soluble product (4). It was also observed that the growth medium, after removal of the fungal cells, possessed the ability to transform 1-naphthol, indicating activity of an extracellular enzyme. Attempts to analyze the radio-labeled material in the aqueous phase gave indications that the radioactivity was associated with a relatively high-molecular weight compound. Therefore, the mixture of a culture filtrate which had been incubated with C^{14}-labeled naphthol was analyzed on a column of Sephadex G-75, and most of the radioactivity was recovered in the first fraction which coincided with the distribution of protein.

In a different experiment the protein fraction of the culture filtrate from *Rhizoctonia praticola* was chromatographed on a column of Sephadex G-200 and fractions obtained that were able to transform 1-naphthol as a substrate (12). The benzene extract of the reaction mixture from such an assay was subjected to mass spectrometric analysis, and major m/e peaks at 144, 286, 428, 570, and 712 were observed. A hypothetical model presenting 1-naphthol polymerization is shown in Figure 2. The compound has a mass of 144 and the detected peaks represent oligomers of the substrate. In subsequent experiments it was possible to separate the individual compounds (2 dimers, 1 trimer and 1 tetramer) by thin-layer chromatography and to establish that they are not fragment ions from higher molecular weight polymers. The extracellular enzyme responsible for the polymerizing activity was isolated, and according to its characteristics it was identified as a laccase (6).

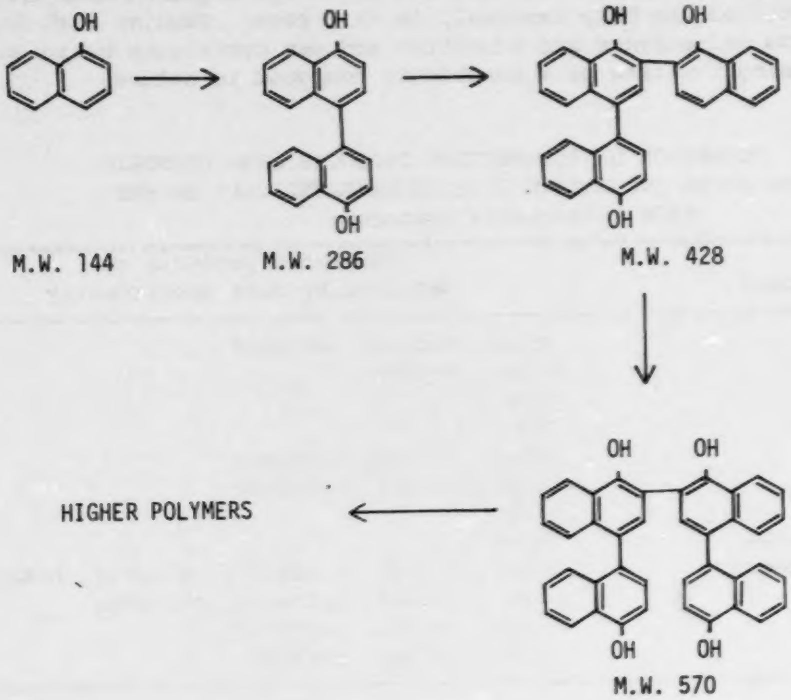

Figure 2. Scheme for polymerization products of 1-naphthol formed by a fungal laccase.

In nature, biological oxidation and coupling of phenols are key reactions that result in formation of products such as lignins, melanins, tannins, alkaloids, and especially humus compounds (7). Therefore, it is not surprising that enzymes capable of these reactions also interact with xenobiotic phenols and it can be assumed that they are incorporated into soil organic matter or sediments in aquatic environments.

The phenol oxidase isolated from the fungus *Rhizoctonia praticola* was also capable of polymerizing other phenolic and naphtholic compounds (Table 3). It could also be shown that the ring-hydroxylated carbaryl metabolites were oxidatively coupled by the fungal enzyme (5). It appeared that these compounds were hydrolyzed to the free naphthol prior to the coupling reaction. The mass spectra of these products indicated that the observed dimers or trimers had undergone further oxidation or dehydration corresponding to loss of H_2 or water (Table 4).

Figure 1 summarizes the various metabolic transformations which were observed in our laboratory with carbaryl. The scheme does not include all the transformations of carbaryl by microorganisms, but it indicates that the metabolic fate of a certain xenobiotic presents a very complex problem. One has to establish all the possible transformation reactions of a chemical in order to foresee the possible formation of potentially hazardous compounds in nature.

The isolation and identification of xenobiotic products from natural environments is a very difficult undertaking, but the knowledge acquired from individual metabolic studies can help immensely in this task. Studies with isolated microorganisms and enzymes are essential and can contribute basic information for determining the fate of a xenobiotic compound in nature.

TABLE 3. FORMATION OF POLYMERIZED PRODUCTS FROM PHENOLIC COMPOUNDS AFTER INCUBATION WITH AN EXTRACELLULAR ENZYME FROM *Rhizoctonia praticola*

Phenolic compound	Enzymatic products as detected by mass spectrometry
Phenol	Dimer, trimer, tetramer
o-Methoxyphenol	Dimer, trimer
p-Methylphenol	Dimer
2,6-Dimethoxyphenol	Dimer
2-Chlorophenol	Dimer, trimer, tetramer
4-Chlorophenol	Dimer, trimer, tetramer
2,4-Dichlorophenol	Dimer
4-Bromo-2-chlorophenol	Dimer
4-Chloro-2-methylphenol	Dimer, trimer, tetramer, pentamer, hexamer
1-Naphthol	Dimer, trimer, tetramer, pentamer
2-Naphthol	Dimer, trimer, tetramer
4-Chloro-1-naphthol	Dimer, trimer, tetramer

TABLE 4. OLIGOMER FORMATION OF CARBARYL METABOLITES

	Monomer	Dimer	Trimer	Tetramer	Pentamer
1-Naphthol	144	286* 284	428*	570*	712
4-Hydroxy-N-methylcarbamate** 1,4-Naphthalene diol	160	314	472 ($-2H_2$) 456 ($-H_2;H_2O$)		
5-Hydroxy-N-methylcarbamate** 1,5-Naphthalene diol	160	300 ($-H_2O$)			

*Isolated by TLC.

**Hydrolysis to free naphthol during oligomerization.

LITERATURE CITED

1. Bollag, J.-M., and S.-Y. Liu. 1971. Degradation of Sevin by soil microorganisms. Soil Biol. Biochem. 3:337-345.

2. Bollag, J.-M., and S.-Y. Liu. 1972. Hydroxylations of carbaryl by soil fungi. Nature 236:177-178.

3. Bollag, J.-M., E. J. Czaplicki, and R. D. Minard. 1975. Bacterial metabolism of 1-naphthol. J. Agr. Food Chem. 23:85-90.

4. Bollag, J.-M., R. D. Sjoblad, E. J. Czaplicki, and R. E. Hoeppel. 1976. Transformation of 1-naphthol by the culture filtrate of *Rhizoctonia praticola*. Soil Biol. Biochem. 8:7-11.

5. Bollag, J.-M., R. D. Sjoblad, and R. D. Minard. 1977. Polymerization of phenolic intermediates of pesticides by a fungal enzyme. Experientia 33:1564-1566.

6. Bollag, J.-M., R. D. Sjoblad, and S.-Y. Liu. 1978. Characterization of a polymerizing enzyme from the soil fungus *Rhizoctonia praticola*. Submitted for publication.

7. Brown, B. R. 1967. Biochemical aspects of oxidative coupling of phenols, p. 167-201. *In* W. I. Taylor and A. R. Battersby (eds.), Oxidative coupling of phenols. Marcel Dekker, Inc., New York.

8. Dorough, H. W., N. C. Leeling, and J. E. Casida. 1963. Nonhydrolytic pathway in metabolism of N-methylcarbamate insecticides. Science 140: 170-171.

9. Kuhr, R. J., and H. W. Dorough. 1976. Carbamate insecticides: chemistry, biochemistry, and toxicology. CRC Press, Inc., Cleveland, Ohio.

10. Liu, S.-Y., and J.-M. Bollag. 1971. Metabolism of carbaryl by a soil fungus. J. Agr. Food Chem. 19:487-490.

11. Liu, S.-Y., and J.-M. Bollag. 1971. Carbaryl decomposition to 1-naphthyl carbamate by *Aspergillus terreus*. Pestic. Biochem. Physiol. 1:366-372.

12. Sjoblad, R. D., R. D. Minard, and J.-M. Bollag. 1976. Polymerization of 1-naphthol and related phenolic compounds by an extracellular fungal enzyme. Pestic. Biochem. Physiol. 6:457-463.

13. Walker, N., N. F. Janes, J. R. Spokes, and P. Van Berkum. 1975. Degradation of 1-naphthol by a soil Pseudomonad. J. Appl. Bact. 39:281-286.

DEGRADATION MECHANISMS

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ABSTRACT

The role of fungi and bacteria in accomplishing the biodegradation of organic compounds is stressed by considering the variety of reactions which these microorganisms can employ to initiate attack on different classes of organic molecules. Reference is made to the value of this type of information, obtained by pure culture studies, in understanding the chemical events which may occur in natural environments, yet at the same time serving only to suggest possibilities rather than to predict specific fates. Among the various classes of compounds considered are linear, branched and cyclic hydrocarbons, aromatic acids, and selected heterocycles. Mechanisms used for the enzymic modification and displacement of different substituents such as sulfonic acids, nitro, alkoxyl, and halide groups are also discussed. Finally, mention is made of the difficulties of assessing which of various degradative reactions may occur with a given substrate by reference to unexpected oxygen-incorporating reactions, alternative degradative routes for the same compounds found in different microbial groups, and the influence which environments can exert on the strategies available for microbial attack.

Bacteria and fungi are the principal agents which accomplish the biodegradation of organic compounds, an important process in the carbon cycle returning carbon to the mineral state for its use in synthesis. Our knowledge of the mechanisms employed by these microorganisms for the dissimilation of different organic compounds derives largely from studies of pure cultures able to grow at the expense of a selected substrate usually at high concentrations and with other conditions optimized principally for the convenience of the investigator. Through such studies mechanisms of degradation have been defined by the characterization of intermediates and enzymes which comprise the overall degradative pathways. In many instances, extensive studies of individual enzymes have revealed the precise details of the mechanisms of the reactions catalyzed. In this account the overall strategies of microbial degradation as revealed by the pathways employed will be emphasized. It might be argued that investigations which yield this information are conducted under conditions which bear little relationship to conditions prevailing in the environment by failure to recognize, for example, that biodegradation occurs naturally in microbial communities at low substrate concentrations. However, it should be pointed out that the objectives of much of this work are frequently biochemical and microbiological, not environmental. Insofar as they reveal the biochemical mechanisms by which different classes of organic compounds are degraded and identify the reactions characteristic of different groups of microorganisms, they provide a valuable basis of information from which to suggest the fates of various environmental pollutants. Only from knowledge of the role of individual microbial species in degrading single organic substrates can one begin to explain the complex relationships which are involved when microbial communities accomplish biodegradation of multiple substrates.

Our understanding of the processes by which microorganisms acquire the ability to degrade novel compounds depends in large part on a background of information about mechanisms of biodegradation. Acclimation, a term used to describe the lag periods during which organisms in water treatment plants gradually "learn" how to degrade some novel chemical, is a phenomenon familiar to most civil engineers. One explanation of this phenomenon is that particular organisms are evolving new degradative abilities. Microortanisms able to utilize organic compounds for growth do so by converting a portion of that compound's structure into compounds which are central to the processes of intermediary metabolism before synthesis of cell constituents can occur. Sufficient energy for synthesis is derived by complete oxidation of the remainder to carbon dioxide and water. For utilization of compounds such as sugars, fatty acids, and amino acids, relatively short pathways of degradation are involved since these compounds bear close structural similarity to intermediary metabolites and are readily converted to such products by enzyme-catalyzed reactions of the type readily found in a standard biochemistry text. Reactions such as retroaldol cleavage, thiolytic cleavage of β-ketoacyl thio-esters and oxidative decarboxylation of α-keto acids are employed to cleave the carbon-carbon bonds of these substrates just as these reactions are characteristic of the metabolism of the same classes of compounds normally present inside the cell. The structures of many other types of compounds such as the natural products of plant biosynthesis are not immediately suitable substrates for reactions of this type and often require extensive structural modification before they can enter central metabolic schemes. Much interesting biochemistry has been discovered from studies of these so-called "peripheral pathways" by which these types of compounds are degraded because appropriate alterations of their structures may involve reactions very different from those of central metabolism. Microorganisms capable of elaborating the necessary enzymes for such sequences are numerous and widespread as seen by their ready isolation from soil and water by selective enrichment procedures. Many such organisms are versatile in this respect, having the ability to utilize many different organic compounds for growth. Others may be unable to derive carbon or energy for growth from a given compound yet may modify its structure, for example, by hydroxylation.

A recent compilation of different microbial transformations of cyclic compounds illustrates the wide array of reactions of which microorganisms are capable (90). Clearly such organisms have acquired the ability to act on natural products and have had a considerable period of time to evolve the necessary enzymes. For those novel chemicals more recently introduced into the environment, and sometimes referred to as xenobiotics, microorganisms have had much shorter periods of time to acquire degradative enzymes. Nevertheless, it is possible to isolate bacteria able to grow with such compounds as the herbicide 2,4-dichlorophenoxyacetic acid, first introduced as an agricultural chemical in about 1945. Evidently the requisite enzymes have been acquired by these bacteria and, according to current views (71), enzymes possessing the appropriate mechanisms have been recruited from pre-existing degradative pathways. Whether these enzymes were previously encoded by the genes of the evolved strain or acquired by an exchange of genetic material their effective functioning in a new setting requires alterations in their specificity, and in the regulatory mechanisms which control their synthesis. As more is learned about how these processes can occur, directed evolution in the laboratory of strains better equipped to degrade specific pollutants becomes a realistic possibility. Attempts to construct such strains will depend on an adequate knowledge of the degradative pathways available in different microorganisms, of the specificities of participating enzymes, and of the mechanisms which regulate enzyme synthesis.

The following is an attempt to identify some of the distinctive features associated with the microbial degradation of different classes of organic compounds and to describe the general nature of certain types of reactions used to effect their catabolism to common intermediary metabolites.

I. ALIPHATIC HYDROCARBONS

The most commonly encountered mechanism employed by bacteria, yeasts and fungi for initiating attack on *n*-alkanes which serve as growth substrates is terminal methyl hydroxylation. For the simplest member of the series, methane, cell-free preparations have been obtained from methylotrophic bacteria which form methanol (27,129). For the longer chain hydrocarbons, two different hydroxylating systems have been described, one from species of *Pseudomonas* which involves three different proteins for activity (118). The other from a species of *Corynebacterium* grown with *n*-octane differs in that cytochrome P_{450} appears to be involved in one of the two fractions required for activity (15). The primary alcohols which result from their action (Fig. 1A) are oxidized via the corresponding aldehydes to fatty acids which can undergo β-oxidation as their coenzyme A thioesters. In some instances, diterminal oxidation can occur to give dicarboxylic acids, apparently by ω-oxidation of fatty acids (96). It has been suggested that 1-alkenes may be intermediates in the process of hydroxylation and the principal evidence for their participation is from anaerobic experiments with a denitrifying organism, *Pseudomonas aeruginosa* (26). A recent report has provided evidence that a *Pseudomonas* growing anaerobically with *n*-decane and nitrate as electron acceptor forms 1-decene as an intermediate en route to 1-decanol (114). There are conflicting opinions, however, about the role of 1-alkenes in the aerobic formation of primary alcohols. They can be excluded as intermediates in the hydroxylation of *n*-alkenes by the hydroxylating systems from *Pseudomonas* and *Corynebacterium* since their action on 1-alkenes leads only to the formation of epoxides (107, 84). On the other hand, evidence for 1-alkene formation from alkanes has also been reported by other groups working with *Nocardia* species (148), *Candida rugosa* (80), and with *Candida tropicalis* (101) suggesting the possibility of an alternative, possibly anaerobic, route.

Mechanisms of *n*-alkane oxidation other than by terminal methyl hydroxylation have been described. A number of organisms are known to form methylketones from alkanes and further metabolism occurs via a Baeyer-Villiger type reaction to give the acetate ester of the alcohol having one carbon less than the substrate (105) (Fig. 1A). The properties of an ester-forming monooxygenase have been described recently (15). Hydrolysis then furnishes the primary alcohol for conversion to a fatty acid and β-oxidation. While formation of ketones is frequently shown as proceeding via the corresponding secondary alcohols, these compounds could be formed readily by ketone reduction rather than by direct hydroxylation of the subterminal methylene group. In the eucaryotic yeasts formation of 1,2-diols from 1-alkenes is logically accounted for by hydration of epoxide intermediates (139). Degradation of the diols in these and other organisms can proceed by oxidation to 2-hydroxy acids, followed by oxidative decarboxylation to give fatty acids one carbon shorter than the parent hydrocarbon (92).

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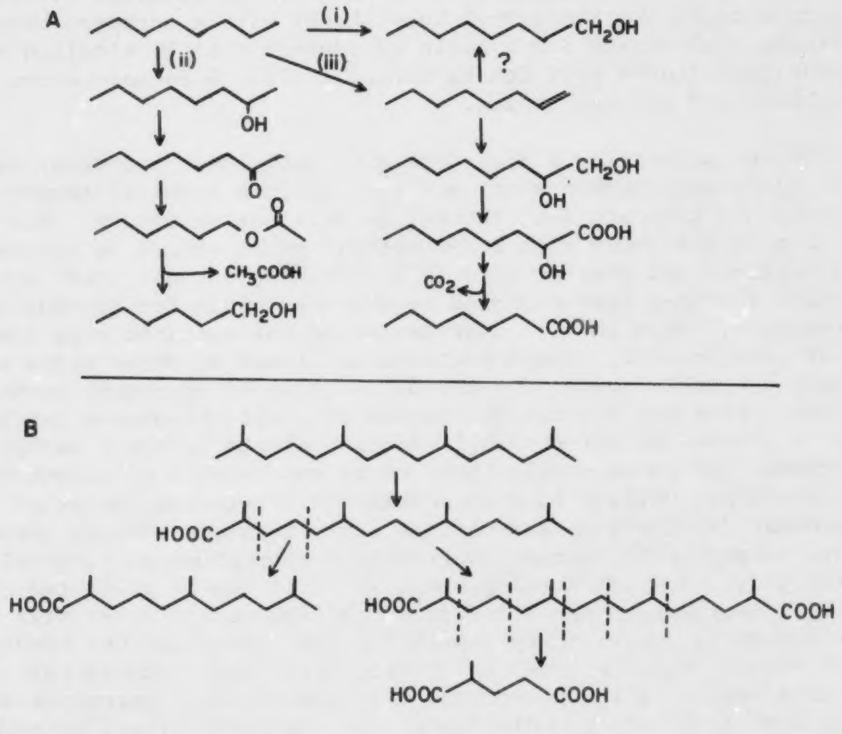


Figure 1. A. Pathways of *n*-alkane degradation in bacteria and yeasts involving (i) terminal methyl hydroxylation, ii) subterminal oxidation to form a methylketone and an acetate ester, iii) formation of 1-alkene and its conversion to a 1,2 diol.
B. Pristane catabolism by a *Brevibacterium* sp. to give mono- and dicarboxylic acids.

Both mono- and diterminal hydroxylation are found as initial reactions in the degradation of branched alkanes such as 2-methylundecane and the diterpene-derived, pristane (2,6,10,14-tetramethylpentadecane), by *Brevibacterium erythrogenes* (120) as shown by the series of mono- and dicarboxylic acids isolated from culture medium. Their structures also reveal that β-oxidation has occurred, removing alternately acetate and propionate residues (Fig. 1B). McKenna and Kallio reached similar conclusions working with strains of *Mycobacteria* (109). Evidently the presence of alpha-methyl substituents in fatty acids does not preclude the same general mechanism of β-oxidation established for unsubstituted mono- and dicarboxylic acids (22, 73). By contrast, the presence of methyl substituents beta to the carboxyl group of acids such as geranoic and citronellic acids does impede β-oxidation as can be seen from a consideration of the route employed by a species of *Pseudomonas* for degradation of the monoterpenes, geraniol and citronellol (132) (Fig. 2A). A modification of the β-oxidation route involves an ATP-dependent carboxylation of the β-methyl αβ-unsaturated fatty acid as its coenzyme A thioester before hydration. This is, in effect, a vinylogous β-carboxylation as distinct from β-carboxylation and is directly analogous to the reaction used in the bacterial degradation of leucine where β-methylcrotonyl CoA is carboxylated (106). The reactions are catalyzed by different enzymes, however (70). After hydration, a substrate for a retroaldol cleavage is formed and its cleavage releases acetic acid, in effect replacing the methyl substituent with a carbonyl group and thereby forming a substrate for a cycle of conventional β-oxidation until another β-methylsubstituted acyl CoA is formed (131). A recent review (119) summarizes these and related topics.

With alkanes possessing a high degree of methyl substitution, as for example where quaternary carbon atoms are present in a terminal *neo*pentyl group, available evidence suggests that incomplete degradation occurs. For example, when an *Achromobacter* grows with 2,2-dimethylheptane attack is initiated at the linear terminus and pivalic acid (2,2-dimethylpropionic acid) accumulates (18). Similar findings were reported by the same group for degradation of *tert*-butylbenzene. Here initial degradation of the aromatic ring leads to the formation of pivalic acid. These patterns of attack at other sites may represent the only available routes for the degradation of compounds containing the *neo*pentyl group possibly because the degree of steric hindrance imposed by the presence of a cluster of three methyl groups prevents terminal methyl hydroxylation. McKenna had shown earlier that organisms capable of growth with *n*-hexadecane could not utilize alkanes possessing diterminal *neo*pentyl groups (108). Somewhat different considerations apply to those alkanes possessing *gem*-dimethyl substituents forming quaternary carbon atoms at internal regions of the chain (e.g., 4,4-dimethylheptane), where it can be predicted that although limited degradation can occur from one terminus, a point will be reached where β-oxidation is prevented by dimethyl substitution because αβ-dehydrogenation cannot occur. Such is true for certain methyl-substituted fatty acids (66). In this regard it is of interest that *gem*-dimethyl compounds such as pantothenic acid (103) and 2,2-dimethylsuccinic acid (135) are readily biodegradable, suggesting that diterminal attack may be an obligatory process for complete degradation of this class of alkanes. The problems of biodegradation of compounds in the environment containing branched alkane functions can best be illustrated by reference to the present use of linear alkylbenzene sulfonates (LAS) detergents in place of the alkylbenzene sulfonates used prior to 1965. This latter group, manufactured using tetrapropylene, contained a significant proportion of compounds having extensive branching in their alkyl sidechains which persisted in the environment causing widespread foam on natural bodies of water. Doubtless the presence in the alkyl sidechains of these detergents of *neo*pentyl and *gem*-dimethyl groups limited their biodegradation for the reasons outlined above providing a rational explanation for their environmental persistence. It must not be assumed, however, that all compounds possessing this type of alkyl branching are similarly persistent. This is manifestly not so as can be shown by noting that the bicyclic monoterpene, camphor, which possesses two quaternary carbon atoms, is a growth substrate for a number of microorganisms (63). Care must be exercised, therefore, to avoid drawing generalized, untenable conclusions about the resistance to biodegradation of a particular structure (77) without reference to the entire molecule in which it is present and to the biodegradation mechanisms available for that class of compounds.

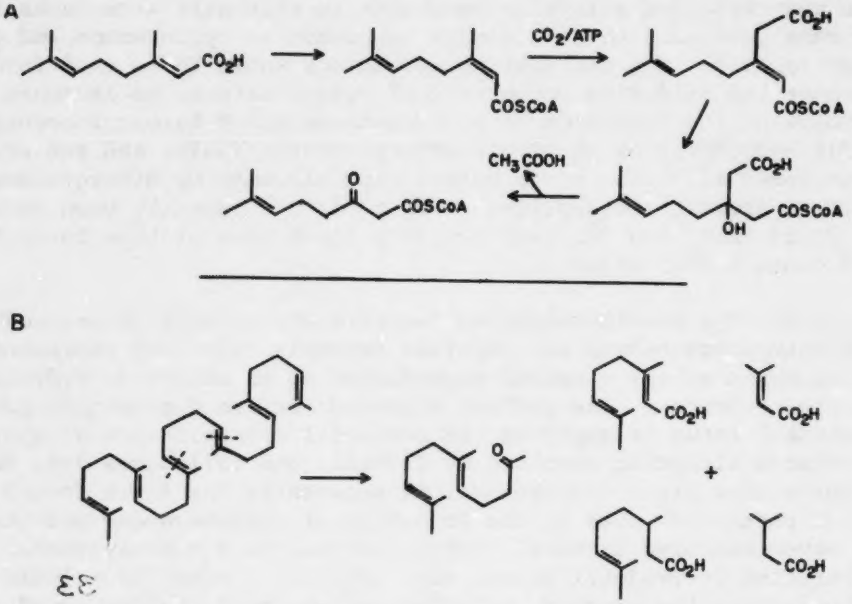


Figure 2. A. Pathway of geranoic acid catabolism by *Pseudomonas citronellolis* involving ATP-dependent carboxylation and cleavage of acetate.
B. Squalene degradation by an *Arthrobacter* sp. showing how cleavage of internal double bonds can form geranylacetone.

In situations where a hydrocarbon is both branched and possesses a number of double bonds, as with the naturally occurring triterpene, squalene, a different type of attack can occur as shown by studies with a strain of *Arthrobacter* able to utilize this compound (155). In place of terminal hydroxylation (or any other of the degradative routes for *n*-alkanes mentioned earlier) this compound undergoes cleavage at its internal double bonds to generate two molecules of geranyl-acetone as shown by isolation of this product from cultures (Fig. 2B). This cleavage is reminiscent of what is apparently a dioxygenase-catalyzed cleavage of β-carotene to vitamin A aldehyde observed with rat liver and intestinal enzymes (61, 112). Other compounds recovered from the *Arthrobacter* cultures included isovaleric, geranoic, citronellic and β,β-dimethylacrylic acids.

The latter conversions while still obscure appear relevant only to that class of hydrocarbons which possesses internal olefinic groups, possibly limited, therefore, to those compounds formed by the pathways of terpene biosynthesis. There is evidence, however, that in some instances double bonds may be introduced at internal positions in *n*-alkanes as shown by the formation of a family of octadecene isomers by a species of *Nocardia* (1).

II. ALICYCLIC HYDROCARBONS AND RELATED COMPOUNDS

To illustrate some of the alternative reaction mechanisms employed by bacteria for the conversion of alicyclic compounds to aliphatic structures reference will be made initially to such simple compounds as cyclohexane and cyclohexane carboxylic acid. The degradative mechanisms known to be used for such compounds include the oxidative conversion of cyclic ketones to lactones followed by hydrolysis, the formation of 1,3-diketones and β-ketoacyl coenzyme A derivatives for hydrolytic or thiolytic attack, respectively, and the aromatization of 6-membered alicyclic rings before ring cleavage by dioxygenases characteristic of aromatic catabolism. This topic has recently been reviewed at length by Perry (115) and for more complete discussion of this subject the reader should consult this source.

The isolation of a biotin-requiring *Nocardia* strain able to use both cyclohexane and methylcyclohexane was reported recently (140) and represents one of the first accounts of the complete degradation of an alicyclic hydrocarbon by pure bacterial cultures. The pathway proposed for its degradation possesses features previously shown to apply in the bacterial dissimilation of cyclohexanol and related alicyclic alcohols by Trudgill and colleagues (39, 62). This is not surprising since cyclohexanol is apparently the first formed intermediate. Of particular note is the formation of cyclohexanone and its conversion to a seven-membered lactone, ε-caprolactone, by a monooxygenase, similar to that purified by Trudgill et al. (38) (Fig. 3). After hydrolytic ring opening of the lactone the product, ε-hydroxycaproic acid, is converted by successive dehydrogenase reactions to adipic acid, ultimately to enter the pool of intermediary metabolites via the pathways used for dicarboxylic acid catabolism (22, 73). Mono-oxygenase reactions leading to the formation of lactones from cyclic ketones appear to be frequently encountered in this area of metabolism as a means of accomplishing ring opening as can be seen from their involvement in the catabolism of cyclopentanol (62), camphor (12), and in the transformations of fenchone (23), progesterone and 17-α-hydroxyprogesterone (51, 117), testosterone (122) and eburicoic acid (100). Such reactions are the biological counterpart of a chemical reaction, the Baeyer-Villiger reaction already referred to in the previous section in connection with methylketone catabolism.

While pure cultures of cyclohexane-utilizing organisms obviously can be isolated (with due attention paid to growth-factor requirements), there is evidence from both laboratory and field studies that degradation of cyclohexane may occur more widely through the agency of microbial communities also provided with with other hydrocarbons. Thus, one organism utilizing a second hydrocarbon provides the ability to hydroxylate cyclohexane to cyclohexanol, the further metabolism of which is assumed by other organisms (8).

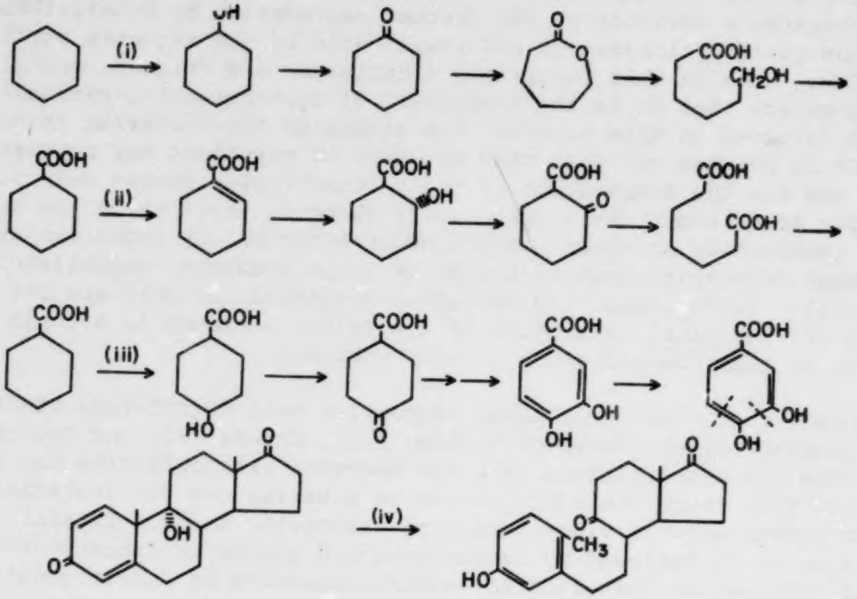


Figure 3. Pathways of bacterial degradation of alicyclic compounds. i) cyclohexane conversion via cyclohexanone and ε-caprolactone to ε-hydroxycaproic acid, ii) cyclohexane carboxylic acid conversion to cyclohexanol-2-carboxylic acid and pimelic acid, probably as coenzyme A thioesters, iii) cyclohexane carboxylic acid aromatization to 4-hydroxybenzoic acid before ring cleavage of protocatechuic acid, iv) aromatization of ring A of 9α-hydroxy-3,17-androstadienedione by aldol cleavage of ring B.

Not shown in Figure 3 is an alternative hydrolytic mechanism of alicyclic ring cleavage which involves hydrolysis of a cyclic β-diketone. By a suitable substitution reaction a hydroxyl group is introduced into the camphor molecule by a soil diphtheroid (now recognized as a strain of *Mycobacterium rhodochrous*) to generate a carbonyl group beta to that already present, thereby permitting hydrolysis of the six-membered ring system (24). A 1,3-diketone is also implicated in the catabolism of *myo*-inositol by *Klebsiella* species formed apparently by dehydrogenation and dehydration reactions allowing hydrolysis of the six-membered ring to give 2-deoxy-5-keto-D-gluconic acid (2) before phosphorylation and aldolase cleavage (3).

For cyclohexane carboxylic acid degradation a related mechanism is employed by a number of bacteria which involves a hydrolytic (or possibly thiolytic) cleavage of a β-keto acyl derivative of coenzyme A. This and the reactions leading to this point are formally those of β-oxidation where the cyclic structure can be viewed as an β-alkyl substituted fatty acid (Fig. 3). This initial formation of a coenzyme A thioester allows αβ-dehydrogenation, hydration and dehydrogenation of the resultant cyclohexanol-2-carboxylic acid derivative to the corresponding ketone. Ring opening then yields pimelic acid as its coenzyme A derivative, for further degradation by β-oxidation or decarboxylation gives cyclohexanone and adipic acid by the sequence outlined earlier (126). Work in this laboratory (Chatterjee and Chapman, unpublished results) suggests that it is the *trans* form of cyclohexanol-2-carboxylic acid which is involved in this pathway in a strain of *Mycobacterium rhodochrous*. It should be pointed out that this sequence of reactions may represent a more general one for the degradation of the related cyclopentane- and cycloheptane-carboxylic acids since these are usually found to serve as carbon sources (either immediately or after a spontaneous mutation) by organisms utilizing cyclohexane-carboxylic acid by the above route (Chapman, unpublished observations; 142). Cyclobutane- and cyclopropane-carboxylic acid are not utilized, however; the bacterial catabolism of the latter compound is a topic to be discussed in another communication in this workshop.

Another route for cyclohexane carboxylic acid degradation studied by three research groups, those of Blakley (10), Kaneda (85) and Trudgill (142), illustrates how aromatization of a six-membered alicyclic ring can occur (Fig. 3). This route therefore cannot be a useful one for degradation of five- or seven-membered ring-containing carboxylic acids. Initial *trans* hydroxylation at C_4 followed by dehydrogenation yields cyclohexanone-4-carboxylic acid and this can be converted to 4-hydroxybenzoate by dehydrogenation reactions. Thereafter, the pathway is that characterized for bacteria utilizing 4-hydroxybenzoate (see next section), namely 3-hydroxylation to protocatechuic acid and either *ortho* cleavage by the organisms studied by Blakley and Kaneda or *meta*-cleavage in that studied by Trudgill and in one organism described by Smith and Callely (134).

The last scheme in Figure 3 shows an aromatization step which occurs when 9α-hydroxyandrostadienedione, an intermediate in steroid degradation, is acted on by strains of *Nocardia* and *Pseudomonas*. In fact, the aromatization occurs as a result of a cleavage of the alicyclic ring B accomplished by another type of ring opening mechanism, an aldolase reaction, the substrate in this case being a vinyl-interrupted aldol (37). The phenol so formed is subsequently hydroxylated to a 3,4-diphenol before its ring is cleaved by a dioxygenase (59).

III. BENZENOID COMPOUNDS

In the previous section it was noted that microbial degradation of certain six-membered alicyclic rings may occur by conversion to benzenoid ring systems. One of the most distinctive features of the reaction sequences used by aerobic microorganisms for catabolism of benzenoid compounds is the utilization of molecular oxygen as a cosubstrate in the cleavage of such rings. For cleavage to occur generally two hydroxyl groups must be present in either an *ortho* or a *para* substitution pattern and for all examples studied this far both atoms of molecular oxygen undergo incorporation into product (68). Such reactions are catalyzed by enzymes known as dioxygenases. Examples of *para*-

substituted diphenols known to be cleaved by enzymes of this type include hydroquinol (99), gentisic (29) and homogentisic acids (21). The sequences of reactions which are initiated by such cleavages, while often mechanistically similar, are generally accomplished by specific enzymes. For *ortho*-substituted diphenols two types of ring cleavage dioxygenases are known. The first group, so-called *ortho* or intra-diol-cleaving dioxygenases, catalyze cleavage of the bond between carbon atoms carrying the hydroxyl substituents. For catechol and protocatechuic acid (3,4-dihydroxybenzoic acid) *ortho* cleavage reactions initiate parallel reaction sequences which converge on a common intermediate, β-ketoadipate enol lactone, and both routes involve lactonization and isomerization steps (Fig. 4). An alternative type of cleavage of 1,2-diphenols (often referred to as *meta*- or extradiol-cleavage, terms which seldom define the precise bond cleaved) is that encountered when the bond adjacent to only one of the hydroxyl groups is ruptured. A wide variety of dioxygenase enzymes catalyzing such reactions has been described and includes those which have as substrates, catechol, 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 2,3-dihydroxy-β-phenyl propionic acid, 1,2-dihydroxynaphthalene and others (19). For catechol and its 3- and 4-methyl homologues the reaction sequences initiated by these enzymes are very similar and may even be catalyzed by single enzymes at least at certain steps (6, 127). The similarity of these reactions prompted an early recognition of their function in terms of a general reaction sequence (32). It can be seen from Figure 5 that generation of 2-keto-4-alkenoic acids either by hydrolysis of ring fission products or by dehydrogenation and decarboxylation of the aldehyde-containing products, allows generation of aldols by hydration and their subsequent cleavage by aldolase reactions. It does not appear to be widely appreciated that the general mechanistic features of both the dehydrogenase and hydrolase routes of this scheme are also found to apply in a number of other reaction sequences initiated by *meta*-cleaving dioxygenases as for example in the bacterial catabolism of homoprotocatechuic acid (136) and of biphenyl (17).

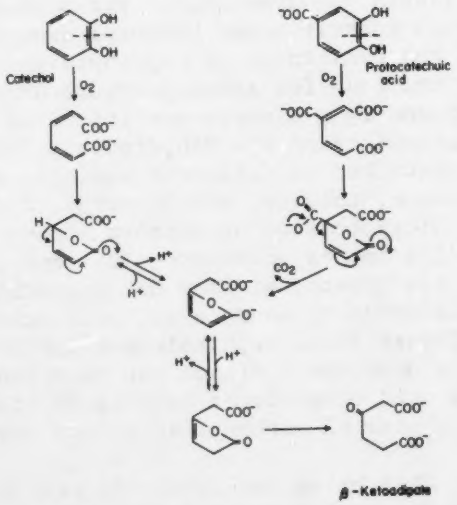


Figure 4. The convergent pathways of *ortho*-fission of catechol and protocatechuic acid leading to β-ketoadipate formation.

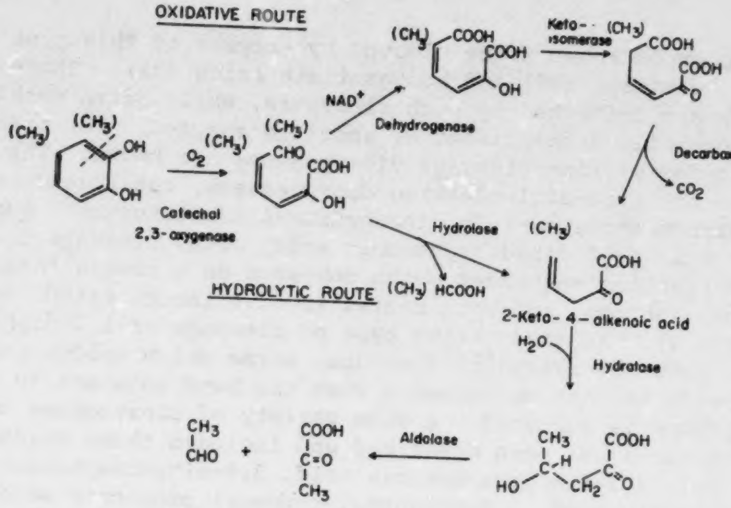


Figure 5. Oxidative and hydrolytic routes which follow *meta*-fission of catechol. The methyl groups in parentheses indicate the principal routes used for 3-methyl- and 4-methylcatechol degradation.

In order to generate suitable substrates for these ring-cleaving dioxygenases, it is frequently necessary for organisms to alter or remove certain substituents and to insert hydroxyl groups in appropriate positions. When these are introduced singly, as usually occurs when an aromatic compound already possesses at least one hydroxyl group, the oxygen for such reactions again derives from molecular oxygen and reactions catalyzed by monooxygenases bring about the introduction of one atom of oxygen into substrate with the other atom undergoing reduction to water. Many enzymes of this type are flavoproteins or require a flavin coenzyme (46). For aromatic compounds not possessing phenolic hydroxyl substituents, hydroxyl groups may be introduced singly, as for example the conversion of L-phenylalanine to L-tyrosine (64) or, as appears more usually the case for aromatic hydrocarbons, two hydroxyl substituents can be introduced by a dioxygenase-initiated mechanism (Fig. 6) which involves the intermediacy of *cis*-dihydrodiols (56). Reactions of this latter type have been described in different bacteria which utilize the aromatic hydrocarbons, benzene, toluene, ethylbenzene, biphenyl and naphthalene, for growth. A similar dioxygenation of benzoic across the C_1-C_2 bond has been shown to operate when *Alcaligenes eutrophus* (125) and other bacteria (124) utilize this substrate for growth; similar dioxygenation mechanisms are thought to function in the degradation of benzene sulfonic acid and various of its alkyl-substituted homologues (14), naphthalene-2-sulfonic acid (93), and aniline (4). By this means dioxygen addition can generate unstable intermediates which lose the sulfonic acid substituent as sulfite, the aromatic amine group as ammonia or facilitate loss of carboxyl as carbon dioxide.

With the knowledge that bacteria usually degrade aromatic hydrocarbons by introducing dioxygen to form 1,2-diphenols and that such diphenols are usually degraded by sequences of reactions of the *meta* fission category referred to earlier, one possesses a framework of information within which a limited number of reasonable predictions can be made about pathways of degradation. A number of examples are available to illustrate this. The formation of benzoic, phenylacetic, atropic and hydratropic acids as intermediates in the degradation of biphenyl (17), diphenylmethane (47), 1,1-diphenylethylene (48), and 1,1-diphenylethane (49), respectively, are compounds all of which could have been predicted as logical intermediates on the basis of previous information. It should be stressed, however, that the predictive value of this information lies in its use in limiting the number of working hypotheses for investigational purposes. The existence of systems which constitute exceptions to the above generalizations points to the need for exercising caution in making dogmatic predictions about how degradation will proceed. For example, the presence of different functional groups in a given compound provides alternative sites for microbial attack, each of which may be characteristic of certain groups of organisms.

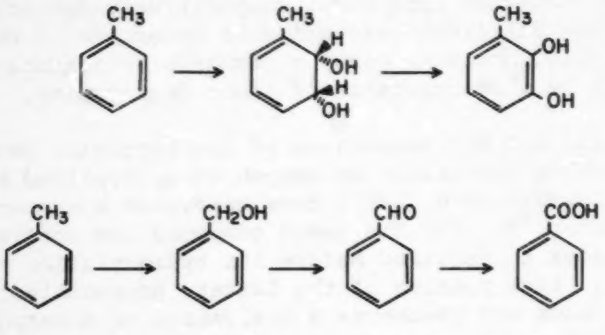


Figure 6. The conversion of toluene to 3-methyl-catechol by addition of dioxygen forming an intermediate *cis*-1,2-dihydrodiol compared with its conversion to benzoic acid by methyl group oxidation.

A number of aromatic hydrocarbons contain alkyl substituents and, as discussed earlier, when such sidechains are extended, linear structures, it is generally the sidechain which is the preferred site of initial attack by *Nocardia* species, thereby forming as intermediates a series of phenylalkanoic acids (35). Compounds with short sidechains may either be attacked in a similar manner or may undergo ring dioxygenation to give 3-alkyl substituted catechols, depending on the microorganism chosen for study (5). Even alkyl sidechains as short as one carbon atom, as for example in toluene, *m*- and *p*-xylene, may be the preferred site of oxidative attack to give benzoic acid and its methyl homologues (Fig. 6). In pseudomonads, degradation of toluene, *m*- and *p*-xylene by this route is a property frequently dependent on the presence of a degradative plasmid designated TOL or XYL depending on its conjugative properties (52, 153). In another example, Kiyohara (91) has shown that phenanthrene is initially attacked by a 1,2-dioxygenation as anticipated but a subsequently formed intermediate, 1-hydroxy-2-naphthoic acid, is itself a substrate for a ring cleavage dioxygenase rather than the anticipated 1,2-dihydroxynaphthalene. Another aromatic compound, 3-hydroxyanthranilic acid, is cleaved by a mammalian dioxygenase despite the fact that it possesses only one hydroxyl group

(111). Alternative *meta* cleavage pathways of alkyl-substituted catechols also have been described. Horvath (75) reported that extracts of a benzoate-grown *Achromobacter* species catalyzed a novel 1,6-cleavage of 4-methylcatechol and other alkyl-substituted catechols. Such reactions imply the existence of *meta* fission pathways different from those outlined in the earlier scheme (Fig. 5), but at this time no further details of this pathway are available.

For more complete accounts of the topic of aromatic catabolism by bacteria the reader is referred to a number of extensive reviews (19, 31, 57).

IV. HETEROCYCLIC COMPOUNDS

A. COMPOUNDS POSSESSING OXYGEN OR SULFUR-CONTAINING RINGS

By comparison with the extensive body of information available on the microbial degradation of aromatic compounds, comparatively few studies have been reported which deal with different categories of heterocyclic compounds. From those which are available, however, certain mechanistic features can be discerned which appear to be characteristic of their degradation.

Studies by Trudgill and his associates of the bacterial catabolism of unsaturated cyclic compounds containing an oxygen atom, typified by such compounds as furan-2-carboxylic acid (146), have uncovered a number of general degradative features (Fig. 7). For the named compound its initial conversion to a coenzyme A thioester is required before its hydroxylation to give the enol tautomer of a lactone. Ring opening of the latter, presumably in the keto form, by hydrolysis yields the coenzeyme A derivative of α-ketoglutaric acid (initially formed as its enol). It should be noted that these reactions are in a sense the complement of those described earlier for such compounds as cyclohexanone and cyclopentanone, i.e., with oxygen already present in the ring the strategy is to make a lactone by introduction of a hydroxyl group. In the earlier examples, the strategy was to make a lactone from a cyclic ketone by insertion of oxygen into the ring. The enzyme system responsible for introducing the hydroxyl group uses water, not oxygen, and can be visualized as catalyzing water addition followed by dehydrogenation. Consistent with this view are the findings that the reaction requires a suitable electron acceptor and proceeds in the absence of oxygen. An essentially similar sequence of reactions is employed by organisms growing with thiophene 2-carboxylate as carbon source (30) (Fig. 7). Here again hydroxylation of the ring is a reaction using the oxygen of water. Reactions of this type are also encountered in organisms able to hydroxylate certain pyridine ring systems (see next section). When the heterocyclic ring is part of a more complex multi-ring system, as for example in dibenzothiophen, then degradation of the other ring systems may represent the first if not the only type of attack. The compounds isolated by Laborde and Gibson (97), when a biphenyl-grown *Beijerinckia* was incubated with dibenzothiophene, include several of those reported by others studying organisms growing at the expense of dibenzothiophene (94). As shown in Figure 7, the most extensive alterations are those in the benzene ring which is dioxygenated and cleaved by the reactions typical of a *meta* fission pathway (Fig. 5). Formation of the sulfoxide is the only indication of a microbial attack on the thiophen ring.

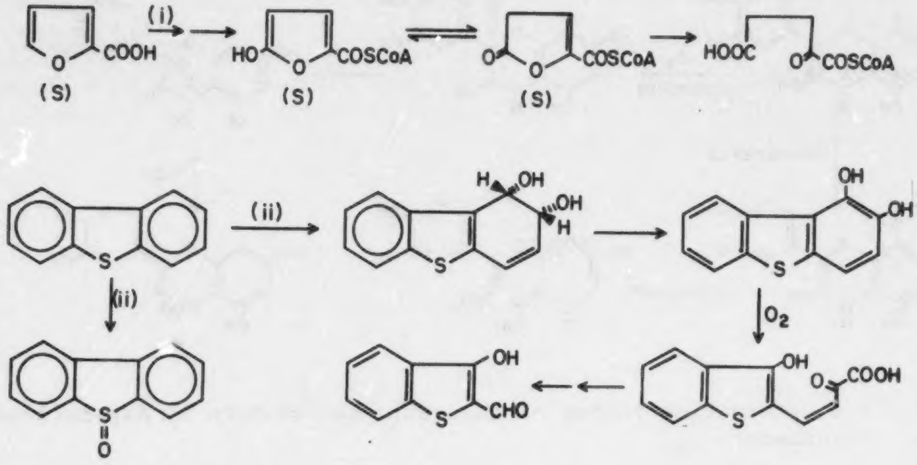
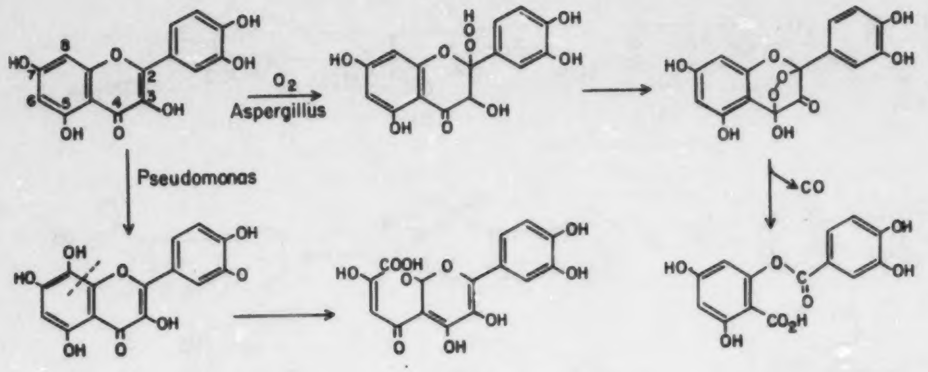


Figure 7. Bacterial degradation of oxygen- and sulfur-containing heterocyclic compounds, i) furoic acid conversion to the coenzyme A derivative of α-ketoglutarate by its hydroxylation at C_5. Shown in parentheses is the position of the sulfur in thiophene-2-carboxylic acid and its metabolites, ii) pathways of dibenzothiophen oxidation by a *Beijerinckia* sp. showing aromatic ring cleavage and sulfur oxidation.

Oxygen-containing rings, which are part of more complex multi-ring systems, may be the initial site of attack as is seen in the degradation of the 3-hydroxyflavone, quercetin, by a strain of *Aspergillus flavus* and other fungi (95) (Fig. 8). The formation of carbon monoxide and the depside shown are consistent with a dioxygenase attack on the pyrone-ring, either via the indicated hydroperoxide or by concerted cyclic peroxide formation. Photosensitized oxygenation of quercetin also forms carbon monoxide apparently via the hydroperoxide route (104). By contrast, the same 3-hydroxyflavone is degraded by *Pseudomonas putida* by initial hydroxylation of the adjacent aromatic ring at C_8 (Fig. 9). The resultant product can now serve as a substrate for a dioxygenase which effects incorporation of an oxygen function in the pyrone ring and which is then believed to undergo a dioxygenase cleavage across C_2-C_3 (130). The related taxifolin, which is an intermediate in the degradation of (+)-catechin by a *Pseudomonas* species, is similarly hydroxylated at C_8 to give 2,3-dihydrogossypetin (82), a substrate for a ring cleavage dioxygenase; however, the structures of later intermediates (81) suggest that the absence of a C_2,C_3 double bond in this series of compounds requires that a degradative sequence differing from that suggested by Schulz et al. (130) is involved.

B. COMPOUNDS POSSESSING N-CONTAINING RINGS

For saturated ring systems containing nitrogen the most usually encountered cleavage mechanism is hydrolysis of the cyclic imine formed by dehydrogenation of the parent compound. The bacterial conversion of L-proline to glutamic semialdehyde (50) and of piperidine-2-carboxylic acid to α-aminoadipic



ure 8. The alternative routes of quercetin degradation by *Aspergillus* and *Pseudomonas* sp.

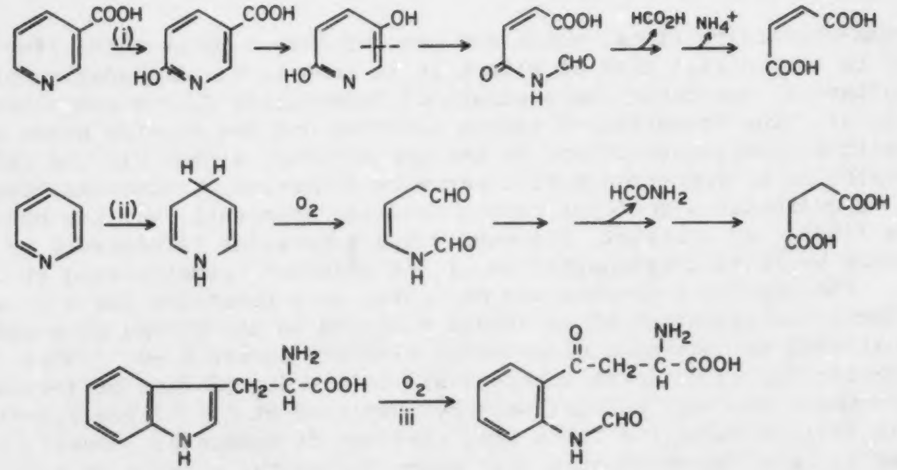


Figure 9. Examples of bacterial degradation of nitrogen-containing heterocycles. i) Nicotinic acid conversion to maleic acid by *Pseudomonas fluorescens* via hydroxylation at C_6. ii) Pyridine degradation by a *Bacillus* sp. involving 1,4-reduction and ring cleavage without prior hydroxylation. iii) Oxygenase cleavage of the pyrrole ring of tryptophan.

semialdehyde (7) are examples of this mechanism. For unsaturated heterocyclic acids such as the pyridine carboxylic acids, nicotinic and picolinic acids, the initial step in their microbial degradation usually involves hydroxylation of the ring in the 6-position with the introduced oxygen function derived from water, not oxygen, in reactions analogous to those found for furan-2-carboxylate catabolism. This initial reaction is found in organisms which degrade nicotinic acid both aerobically and anaerobically from such genera as *Pseudomonas*, *Arthrobacter* and *Clostridium*. The routes taken after this reaction may vary with the organism and growth conditions. The pathway of nicotinic acid degradation elucidated for *Ps. fluorescens* (9,78) is shown (Fig. 9). It can be seen that a second hydroxyl group is introduced, this time by a monooxygenase mechanism displacing the carboxyl before ring cleavage yields an amide derivative of maleic acid and ultimately fumaric acid. A number of organisms able to utilize nicotine employ a similar water-dependent hydroxylation mechanism to form 6-hydroxynicotine (72). Once substituted with one hydroxyl group, however, pyridine compounds generally undergo further hydroxylation via monooxygenase-type mechanisms to provide suitably substituted substrates for ring cleavage (46). With pyridine itself (150) and also of an N-methylpyridinium derivative, 4-carboxy-N-methylpyridine hydrochloride, formed photochemically from Paraquat, no hydroxylated intermediates have been detected. Instead, an initial 1,4-reduction reminiscent of pyridine nucleotide reduction appears to be necessary, yielding 1,4-dihydro-pyridine derivatives. Evidence supports a cleavage mechanism by direct dioxygenation of one of the double bonds. In Figure 9 is shown the pathway proposed for pyridine degradation by a species of *Bacillus*.

There is a marked similarity between this type of ring opening mechanism and that catalyzed by the dioxygenase, L-tryptophan pyrrolase (Fig. 9). Later steps in pyridine breakdown by this organism yield ammonia, formate and succinate. A different type of ring opening has been described for indole degradation in which a Gram-positive coccus appears to form 2,3-dihydroxy indole before its intradiol cleavage. Evidence to suggest the participation of a dihydrodiol intermediate is provided by the indentification of 2-keto-3-hydroxyskatole from skatole, the 3-methyl-substituted homologue of indole (53).

V. REACTIONS OF SELECTED FUNCTIONAL GROUPS

Another way of evaluating the degradative reactions which an organic compound may undergo is to consider the different functional groups in the compound in relation to what is already known of microbial action toward such groups. Selected functional groups will be considered here with a number of examples chosen to illustrate their degradation.

i) ETHERS

The presence of an O-alkyl ether derivative of a phenyl in a given compound represents one site where microbial action may be initiated. From studies with a number of O-methyl ethers of aromatic compounds, many having structures related to lignin monomers, it is now recognized that aerobic microorganisms can effect demethylation by monooxygenation to form what is probably the unstable hemiacetal of the parent phenol and formaldehyde. Such an intermediate would then yield spontaneously the phenolic and formaldehyde products observed (128,144) (Fig. 10).

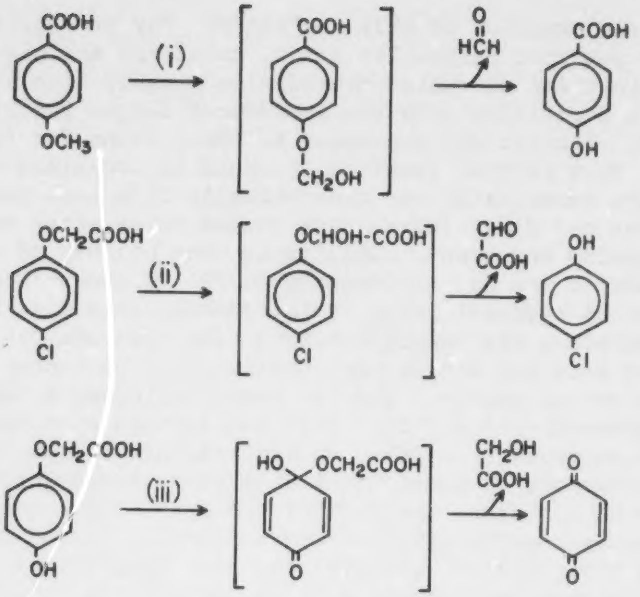


Figure 10. The bacterial cleavage of phenolic ethers. i) The conversion of *p*-anisic acid to formaldehyde and *p*-hydroxybenzoic acid by formation of the intermediate hemiacetal. ii) Formation of glyoxylate from *p*-chlorophenoxyacetic acid by a monooxygenative formation of a hemiacetal. iii) Formation of glycollic acid and *p*-benzoquinone from *p*-hydroxyphenoxyacetic acid via the intermediate hemiketal.

Such reactions also explain the cleavage of the ether sidechain of phenoxyacetic acids as with the degradation of chlorinated herbicides such as 4-chlorophenoxyacetic (43) and 4-chloro-2-methylphenoxyacetic acids (55). In this instance, glyoxylate is formed together with the parent phenol (Fig. 10). Fortuitous monooxygenation of 4-hydroxyphenoxyacetic acid at C-1 by an enzyme from *Pseudomonas acidovorans*, which has 4-hydroxyphenylacetic acid as its normal substrate, can also accomplish cleavage of the ether bond to yield glycollic acid and 1,4-benzoquinone (67) (Fig. 10). A number of examples have been described, however, which show that aromatic O-methyl substituents are not always subject to this type of attack but may keep this structure intact until aliphatic products are formed where they may now be present as methylesters (as with syringate and 3-O-methylgallate degradation by *Pseudomonas putida* [141]) or as ethers of enols (as is the case for degradation of methoxyl-substituted gentisates and their precursors) (102; Chapman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q90, p. 276). Hydrolysis and hydration reactions can then liberate the methyl moiety as methanol.

Studies of the microbial degradation of strictly aliphatic ethers have not been extensive; the mechanisms for cleavage of such ether bonds are not yet clear. In one study, however, investigation of the microbial degradation of carboxymethoxy-succinate (CMOS), a potential replacement of phosphate as a detergent builder, has revealed a mechanism which is formally a β-*trans*-

elimination of glycollic acid forming fumaric acid (116) (Fig. 11). Other β-eliminations of this type are known for the microbial depolymerization of β-1,4-polymers of uronic acids, such as alginic acid (123). Microbial degradation of a related compound, carboxymethoxymalonic acid (Builder M) occurs apparently via a reductive type of cleavage to yield glycollic and malonic acids (Gledhill, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, Q65, p. 201; personal communication). Bacteria capable of utilizing ethylene glycol and its various polymers have been described (83,87), but there is presently no clear indication as to how the ether bonds of the polymers are cleaved.

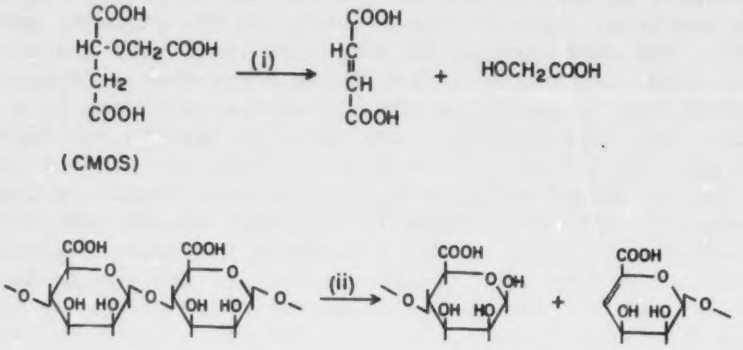


Figure 11. Cleavage of aliphatic ethers by a β-elimination mechanism. i) Elimination of glycollic acid from carboxymethoxysuccinate (CMOS). ii) Depolymerization of alginic acid by a β-eliminase.

In the case of thio-ethers, microorganisms are known which oxidize the sulfur of such functional groups to the sulfoxide. This is found both for methyl thio-ethers (98) as well as with compounds containing sulfur in heterocyclic rings such as *d*-biotin (156) and the earlier mentioned dibenzothiophen (97).

ii) HALIDES

The subject of microbial dehalogenation has been dealt with in recent reviews (20,60). Since many of the chemicals, which persist in the environment and which may undergo biomagnification to toxic levels, are organohalides, a general view has developed that all halogen-containing compounds are of industrial origin and are potentially harmful. Currently at least three hundred different halogen-containing compounds of naturall occurrence are known (133) and many of these are of marine origin. Admittedly this represents only a small fraction of the total range of naturally synthesized organic compounds. Nonetheless microorganisms can be isolated which modify or completely degrade both natural and synthetic organohalides. Table 1 shows some of the types of enzyme reactions known by which organohalides can lose halide. For additional details the reader is referred to the excellent review by Goldman (60). Reactions such as these have been studied usually in bacteria capable of growth with a particular halogenated substrate. Other reactions are known, however, in which dehalogenation occurs fortuitously because halogenated compounds can serve as substrates for enzymes normally acting on non-halogenated substrates and halide is labilized as a consequence of the reaction mechanism. One example of microbial utilization of an organohalide will be discussed to show how dehydrohalogenation can account for halide mineralization. When *Pseudomonas* and *Arthrobacter* strains degrade 2,4-dichlorophenoxyacetic acid, 3,5-dichlorocatechol is an intermediate which undergoes an *ortho*-dioxygenative ring cleavage (Fig. 12). Lactonization then yields the doubly unsaturated lactone whose formation is most reasonably formulated as proceeding via the lactone in square brackets. Loss of the elements of HCl probably occurs spontaneously (44,143). The enol lactone is then hydrolyzed to a chlorine-substituted maleylacetic acid. Recent work from these laboratories (Keenan and Chapman, unpublished observations) has shown that the next step is a reductive one requiring $NADH_2$, but interestingly, two moles of reduced pyridine nucleotide are consumed per mole of chloromaleylacetic acid; the product is β-ketoadipate and chloride is formed quantitatively. Since a similar action is observed by maleylacetate reductase, an $NADH_2$-dependent enzyme reducing maleylacetate to β-ketoadipate which is found in bacteria degrading resorcinol (25), it appears that such an enzyme may have been recruited for use in dehalogenation. A possible explanation for the experimental observations is suggested in Figure 12 where the first formed reduction product is shown as β-eliminating HCl to generate the related maleylacetate which can serve as substrate in a second reduction to give β-ketoadipate.

TABLE 1. REACTIONS USED BY MICROORGANISMS FOR HALIDE REMOVAL

I	Substitution	a) Hydrolytic b) Neighboring group attack
II	Elimination	Dehydrohalogenation
III	Reduction	Reductive Dehalogenation

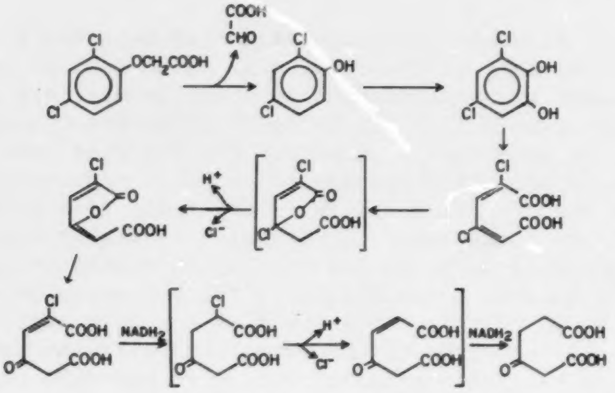


Figure 12. Pathway of 2,4-dichlorophenoxyacetic acid degradation showing how lactone formation can eliminate chloride and how reduction can lead to loss of second chloride substituent.

iii) NITRO- AND SULFONIC ACID SUBSTITUENTS

A number of industrial chemicals contain nitro- and sulfonyl-functional groups. Reactions for the removal of these groups have been described in microorganisms utilizing compounds containing these functional groups where it appears that these groups must be removed in early steps before the remainder of the molecule can be utilized.

Recent work with organisms able to assimilate nitroalkanes has revealed the presence of a dioxygenase which converts two molecules of 2-nitropropane to acetone and nitrite (89). This mechanism appears to be one specific for nitroalkanes and is distinct from the oxidase type of attack observed when D-amino acid oxidase acts on nitromethane or nitroethane (121) or the release of nitrite effected by the action of glutathione S-transferase (65). Many aromatic nitro compounds can undergo microbiological reduction to amino and hydroxylamino products, but reduction does not appear to be the principal route by which aromatic nitro compounds such as p-nitrobenzoic acid are degraded by *Nocardia* species (16). The first reaction is removal of the nitro group usually as nitrite and its replacement by a hydroxyl group (Fig. 13A). Whether this occurs by a hydrolytic or, as appears more likely, an oxygenase-type reaction is not established because of difficulties in obtaining active cell-free enzyme preparations. An oxygen-dependent enzyme system capable of converting *p*-nitrophenol to hydroquinone and nitrite has recently been obtained in Dr. D. T. Gibson's laboratory (Spain and Gibson, personal communication), suggesting that oxygenative removal of aromatic nitro groups is likely to be encountered elsewhere.

The linear alkyl benzene sulfonate (LAS) detergents represent one important class of aryl sulfonates used on a large scale. Studies of their microbial dissimilation have tended to concentrate largely on the degradation of the alkyl sidechains since it is this process which rapidly alters the detergent property of these molecules. Loss of the sulfonyl group evidently takes place as can be shown by formation of inorganic sulfur-containing products and disappearance of the UV-absorbing aromatic ring in pure microbial cultures and in microbial communities. One way in which this can occur is evidently by the displacement of the sulfonic acid group by a single hydroxyl group as found in the degradation of alkylbenzene sulfonates by a species of *Bacillus* (152) (Fig. 13B). It is not yet clear whether this is a monooxygenase-type reaction as appears likely. Fungi apparently can desulfonate sulfophenylalkanoic acids in a reductive manner (151). An alternative mechanism for sulfonic acid group removal has been identified in studies on the degradation of benzene-, toluene- and naphthalene-sulfonic acids (14,93). Here, a dioxygenase mechanism appears to be involved, similar to that noted earlier for the dioxygenation of benzoic acid. Addition of dioxygen to form a 1,2-diol where the sulfonic acid group is also attached to one of the oxygenated carbon atoms (Fig. 13C) constitutes a feasible mechanism for the observed loss of sulfite and formation of a 1,2-diphenol. Unlike the pathway of benzoic acid degradation referred to, no dehydrogenase step is required here; spontaneous elimination accomplishes aromatization directly.

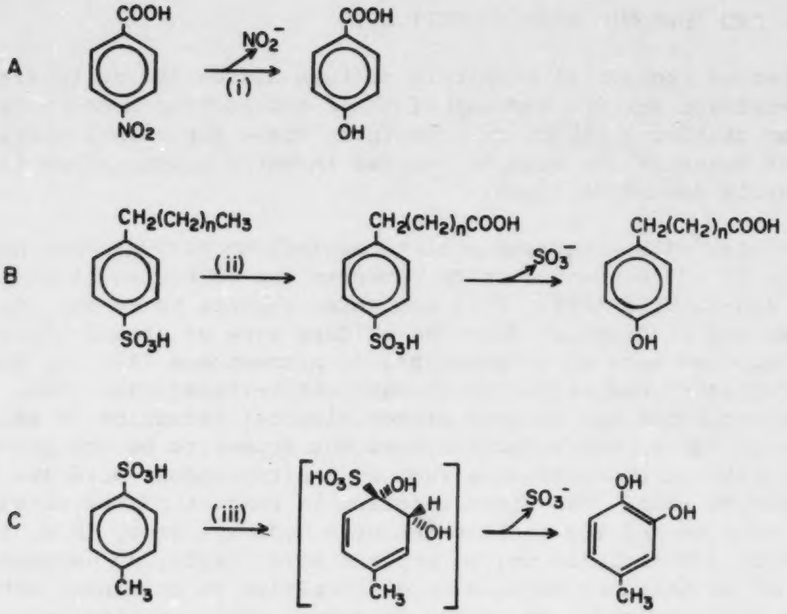


Figure 13. Elimination of nitro and sulfonic acid substituents. i) Displacement of a nitro group from *p*-nitrobenzoic acid to form nitrite. ii) Removal of a sulfonic acid group from a sulfophenyl alkanoic acid metabolite of a linear alkylbenzene sulfonate. iii) Sulfite formation by dioxygenative attack of *p*-toluene sulfonic acid.

iv) AROMATIC AMINES AND DERIVATIVES

A wide variety of aniline derivatives are synthesized for use as herbicides. The derivatives may be variously substituted in the aromatic ring with alkyl, alkoxyl and halide groups and the aniline nitrogen derivatized either as an acyl anilide, a carbamate or a phenylurea. Three examples of named herbicides based on 3,4-dichloroaniline are shown in Figure 14. Bacteria and fungi capable of modifying these structures have been described (86). One general mechanism which may be employed is that whereby cleavage of the carbon-nitrogen bond liberates the parent aniline together with aliphatic products. An acylamidase enzyme system possessing a broad substrate specificity and capable of hydrolyzing examples of all three classes of these herbicides has been purified from a strain of *Bacillus sphaericus* (41). For N-alkyl-substituted phenylureas, an alternative mechanism of degradation involves one or more successive demethylation or demethoxylation reactions as necessary to yield the parent phenylurea (147).

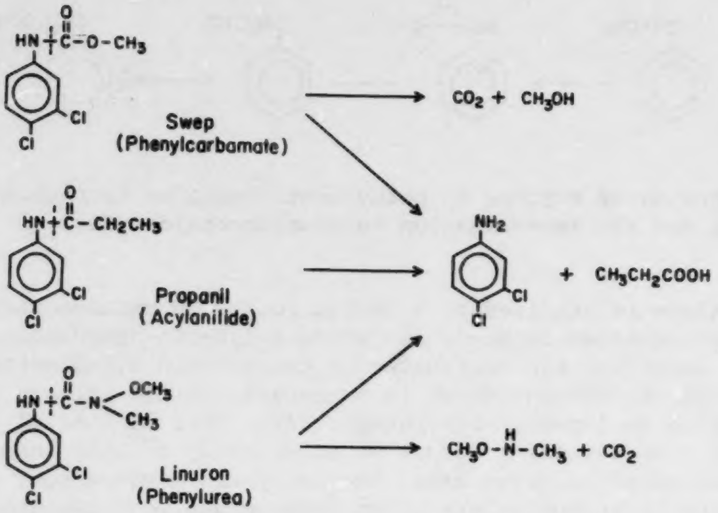


Figure 14. Degradation of aniline-based herbicides. Cleavage of carbamate-, anilide-, and phenylurea-herbicides to yield the parent aniline by an amidase-type reaction.

VI. SOME DIFFICULTIES IN PREDICTING DEGRADATIVE ROUTES

Having outlined some of the mechanisms employed by microorganisms for degradation of different classes of organic compounds and for their attack on a selected range of functional groups, some specific examples will be considered here to illustrate a few factors which may influence or modify the degradative routes which are used. These examples should further serve to illustrate the difficulties of predicting routes of degradation.

It was stated earlier that aromatic hydrocarbons may be degraded by microorganisms which either oxidize alkyl sidechains or introduce dioxygen into the ring to form *cis*-diols. Consideration of the degradation route of the aromatic hydrocarbon, styrene, by bacteria which grow with this compound reveals that the former possibility is realized in an unexpected manner. Recent results from this laboratory (Battles and Chapman, unpublished observations) implicate styrene oxide as the first formed intermediate (Fig. 15) which subsequently undergoes isomerization to phenylacetaldehyde; thereafter its dehydrogenation furnishes phenylacetic acid. While this conversion is equivalent to sidechain oxidation, it illustrates that bacteria, like fungi and higher organisms (34), are also capable of forming epoxides from certain hydrocarbons. Isomerization (rather than hydration as occurs with arene oxides in animal tissues [110]) appears to be the reaction used here for further degradation. A number of cyclic ketones known to be formed from such chemicals as the cyclodiene insecticides appear to be similarly formed by isomerization of precursor epoxides.

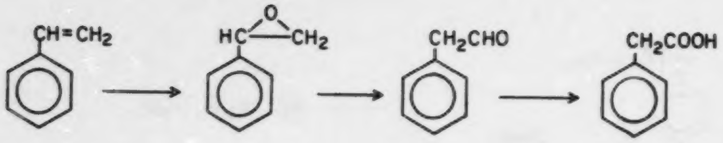


Figure 15. Conversion of styrene to phenylacetic acid by formation of styrene oxide and its isomerization to phenylacetaldehyde.

When naphthalene is utilized as a carbon source by pseudomonads and other bacteria the first reaction is one which forms a 1,2-*cis*-dihydrodiol (Fig. 16) (56). When such organisms are confronted by the related 1,5-dimethyl-naphthalene it is a methyl substituent which is oxygenated to the primary alcohol and thereafter converted to 1-methyl-5-naphthoic acid (36); neither of the aromatic rings is oxidized. In a similar situation under study in this laboratory (Tam and Chapman, unpublished observations), the tricyclic hydrocarbon, acenaphthene, is converted by a naphthalene-grown *Pseudomonas* to 1-acenaphthenol and then to 1-acenaphthenone (Fig. 16). Here, the site of oxygenation is the methylenic carbon of the five-membered ring rather than the aromatic ring system probably because it is the site of highest electron density and preferred by an electrophilic species of oxygen. The related acenaphthylene (not shown in Fig. 16) possesses a double bond in the five-membered ring and it is this group which is also the site of attack by naphthalene-grown cells, forming the 1,2-*cis*-dihydrodiol presumably by a dioxygen addition. It seems clear that the naphthalene dioxygenase is responsible for this unexpected attack on the five-membered ring, giving either mono- or dioxygenated products depending upon the structure involved. These selected examples illustrate how a dioxygenation reaction can form oxygenated products which on purely steric grounds would not be anticipated. Others are known such as the 2,3- or *meta*-cleavage of 3-methyl- and 3-methoxy-catechols by catechol 1,2-dioxygenase (54,76), the formation of *o*-phthalide from 2-methylbenzoic acid by a benzoate-1,2-dioxygenase (79) and the formation of small amounts of 1-phenylethanol from ethylbenzene apparently through the action of the enzyme system which forms 3-ethylcatechol as the principal product (58). There are also examples to show that monooxygenase enzymes may also act to form products which would not be expected from purely steric considerations. The formation of 4-hydroxymethylphenylalanine, of 3-hydroxy-4-methyl-phenylalanine and of 3-methyltyrosine by the action of L-phenylalanine-4-hydroxylase on 4-methylphenylalanine (33) can be rationalized only from consideration of the nature of the reaction mechanism and the form of "active" oxygen involved. Substituents other than methyl, such as chlorine or bromine, can also undergo migration as is found when certain halogenated aromatic compounds are acted upon by those hydroxylating enzumes which act by forming arene oxide intermediates (34). It is the rearrangement of such epoxides which underlies this migration of substituents and which is known as the "NIH shift."

It should not be assumed that the introduction of hydroxyl groups into non-heterocyclic compounds is invariably an oxygen-dependent process. The conversion of *p*-cresol to 4-hydroxybenzylalcohol by species of *Pseudomonas* is a reaction which uses the oxygen of water as the source of the hydroxyl group

(74). The mechanism appears to involve formation of a quinone methide intermediate by a dehydrogenation reaction followed by hydration. This mechanism also accounts for the strict specificity of this hydroxylation in acting only on 4-methyl-substituted phenols. A similar reaction involving a quinone methide intermediate has been described in the degradation of α-conidendrin by a *Pseudomonas* species (145).

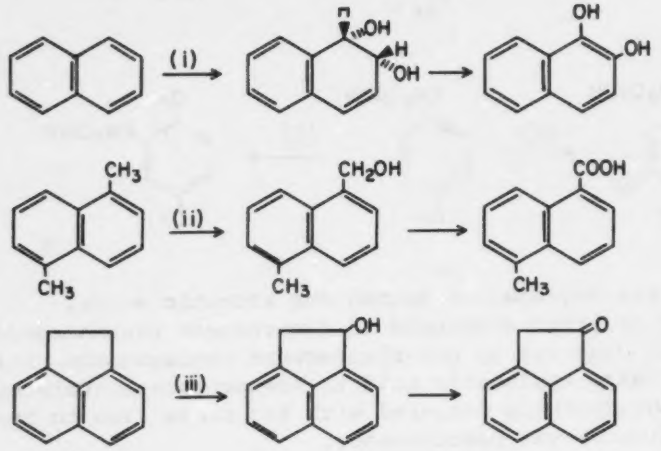


Figure 16. Conversion of naphthalene and related compounds to oxygenated products. i) Formation of 1,2-dihydroxynaphthalene from naphthalene via *cis*-naphthalene 1,2-dihydrodiol by a naphthalene-utilizing *Pseudomonas* sp. ii) Conversion of 1,5-dimethyl naphthalene to 1-methyl-5-naphthoic acid via 5-hydroxymethyl-1-methylnaphthalene. iii) Formation of 1-acenaphthenol and 1-acenaphthenone from acenaphthene.

The realization that frequently there are a number of alternative routes by which a given chemical may be degraded by bacteria and fungi has prompted examination of the possibility that such differences may be uniquely characteristic of different microbial groups. This has proved to be true for procaryotic and eucaryotic groups within the microbial world, for different genera and even for different species within the same genus.

Within the genus *Pseudomonas* two different mechanisms for the cleavage of protocatechuic (3,4-dihydroxybenzoic) acid are known and are useful in taxonomic work (138); the *ortho* or 3,4-cleavage appears characteristic of fluorescent pseudomonads while the 4,5-(*meta*)-cleavage is found in the non-flourescent or acidovorans group (Fig. 17A). Curiously, the subsequent reactions of the degradative sequences initiated by these dioxygenase cleavages appear to be present in both groups of organisms (113,137). A third dioxygenase cleavage of protocatechuic acid, across the 2,3-bond, has been described in certain species of *Bacillus* (28).

Another feature which distinguishes the fluorescent and non-fluorescent pseudomonads is the route of degradation of 4-hydroxyphenylacetic acid. Fluorescent pseudomonads form homprotochatechuic (3,4-dihydroxyphenylacetic) acid, whereas non-fluorescent members of this group convert it to homogentisic acid by a novel hydroxylation which effects migration of the carboxymethyl sidechain (Fig. 17B) (67,136).

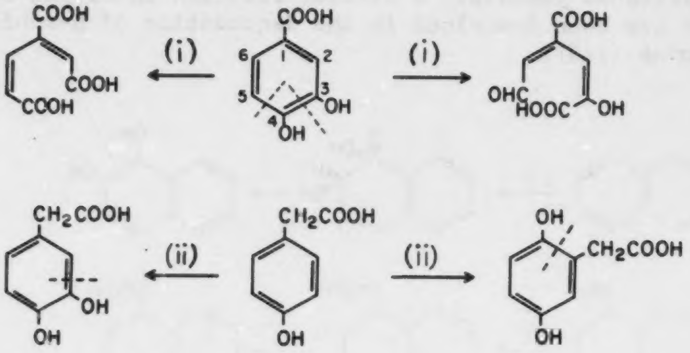


Figure 17. Alternative degradative routes for aromatic acids. i) *Ortho* cleavage of protocatechuate in fluorescent pseudomonads compared with *meta* cleavage by non-fluorescent pseudomonads. ii) Conversion of *p*-hydroxyphenylacetic acid to homoprotocatechuate by fluorescent pseudomonads compared with its conversion to homogentisate by non-fluorescent pseudomonads.

To illustrate generic differences, reference will be made to the degradation of 4-hydroxybenzoic acid and its 3-methoxy-substituted derivative (vanillic acid). Members of the genus *Pseudomonas* convert these compounds to protocatechuic acid either by direct hydroxylation or demethoxylation respectively. Cleavage of protocatechuic acid will occur either 3,4- or 4,5- depending upon the pseudomonas group involved (Fig. 18). While certain of the above reactions may also be found in members of the genus *Bacillus*, other members of this group can effect a novel hydroxylation-facilitated carboxyl migration of 4-hydroxybenzoic acid and vanillic acid not yet found in other groups (88). A consequence of this is that gentisic acid and its 4-methoxyl-derivative are now the respective products; they are subsequently degraded by pathways completely different from the protocatechuic acid routes (Fig. 18).

One example will suffice to show how fungi and bacteria act in very different ways on the same chemical. The herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), is utilized for growth by some bacteria and the principal reaction sequence used appears to be that shown (Fig. 19), namely, loss of the sidechain and hydroxylation at C_6 before ring cleavage. The sequence described earlier (44) (Fig. 12) shows the continuation of the pathway. By contrast, the fungus, *Aspergillus niger*, which does not utilize 2,4-D for growth, introduces hydroxyl groups both at C_5 and C_4, the latter hydroxylation causing a migration of chlorine to an adjacent position (Fig. 19) (45).

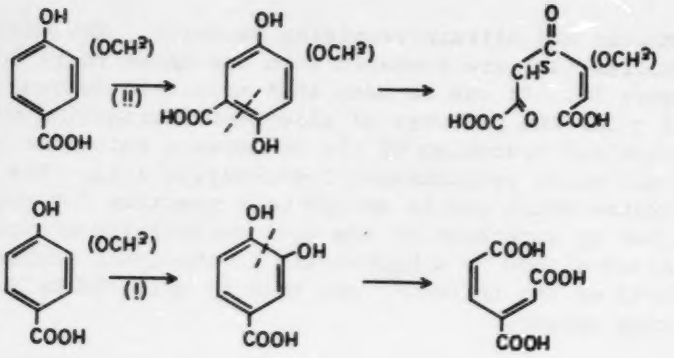


Figure 18. Degradation of 4-hydroxybenzoic and vanillic acids by species of *Pseudomonas* and *Bacillus*. i) Formation of protocatechuate by hydroxylation and demethoxylation reactions before ring cleavage in pseudomonads. ii) Formation of gentisate and 4-methoxygentisate by carboxyl migration facilitated by hydroxylation as found in species of *Bacillus*.

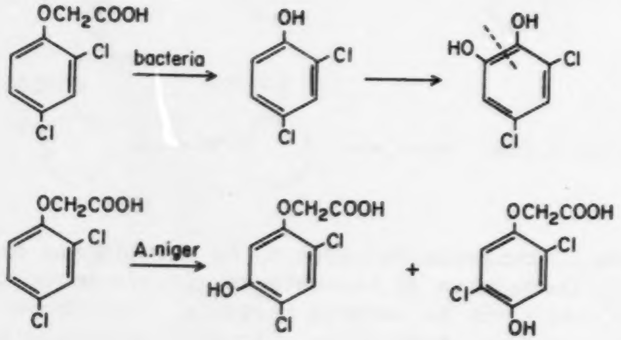


Figure 19. Action of bacteria and fungi on 2,4-dichlorophenoxyacetic acid (2,4-D). Formation of 3,5-dichlorocatechol by sidechain loss and ring hydroxylation by bacteria compared with fungal hydroxylation at C_4 (with chlorine migration) and at C_5.

Lastly, mention should be made of the fact that while much of the foregoing discussion has described degradative sequences which use molecular oxygen at one or more steps, many of the environments into which natural and synthetic compounds are introduced are anaerobic. Degradation must occur therefore by catabolic routes which are independent of oxygen, i.e., by anaerobic photometabolism, by fermentation and by anaerobic respiration. Few detailed studies of such types of degradation have been reported. The anaerobic degradation of benzoate, however, has been the subject of extensive studies, principally by the research group of Evans (42) who has studied its dissimilation by both photosynthetic and nitrate-respiring bacteria. The general features of its photometabolism (40) are compared with one known route for its aerobic catabolism in Figure 20. It can be seen that anaerobic degradation of this aromatic compound joins the pathways of alicyclic degradation outlined earlier (Fig. 3). Reduction and hydration of the coenzyme A thioester of benzoate occurs to give the alicyclic cyclohexanol-2-carboxylic acid. The strategy is to generate a ring system which can be opened by a reaction not requiring oxygen. This is accomplished by formation of the cyclohexanol-2-carboxylic acid derivative which is cleaved either by a hydrolytic or thiolytic mechanism. The resulting pimelic acid or its thioester can then be oxidized by using electron acceptors other than oxygen.

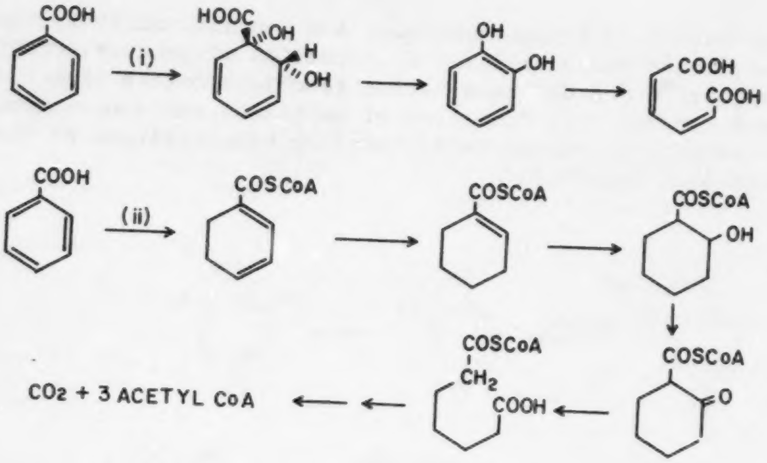


Figure 20. Comparison of benzoate degradation by aerobic and anaerobic bacteria. i) Conversion of benzoate to *cis,cis*-muconate by oxygen-dependent reactions by aerobic bacteria. ii) Conversion of benzoate by anaerobic bacteria to alicyclic compounds by reduction and hydration permitting ring cleavage in the absence of oxygen.

Recent reports indicate that aromatic compounds such as phenol and catechol are degraded by anaerobic communities of methane-forming organisms (69). The reactions by which these conversions occur have not yet been elucidated.

Much of our knowledge of the mechanisms employed by microorganisms for the dissimilation of organic compounds derives from studies with organisms isolated from terrestrial and freshwater environments. Few studies have been reported of the mechanisms of degradation of organic chemicals by marine organisms in marine environments. It remains to be established whether those groups of microorganisms currently recognized as playing important roles in degradative processes in soil and freshwater are such important agents in brackish, estuarine and marine waters. There is some evidence to suggest that fresh water organisms may not be able to effect degradation of such compounds as nitrilotriacetic acid (11) or may be inhibited in their degradation of hydrocarbons (149) in saline environments. It is evident, therefore, that continuing research on biodegradation of organic compounds should address these and other questions so as to amplify our present knowledge of these processes in marine environments.

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LITERATURE CITED

1. Abbott, B. J., and L. E. Casida. 1968. Oxidation of alkanes to internal monoalkenes by a *Nocardia*. J. Bacteriol. 96:925-930.

2. Anderson, W. A., and B. Magasanik. 1971. The pathway of *myo*-inositol degradation in *Aerobacter aerogenes*. Identification of the intermediate 2-deoxy-5-keto-D-gluconic acid. J. Biol. Chem. 246:5653-5661.

3. Anderson, W. A., and B. Magasanik. 1971. The pathway of *myo*-inositol degradation in *Aerobacter aerogenes*. Conversion of 2-deoxy-5-ketogluconic acid to glycolytic intermediates. J. Biol. Chem. 246:5662-5675.

4. Bachofer, R., F. Lingens, and W. Schafer. 1975. Conversion of aniline into pyrocatechol by a *Nocardia* sp.; incorporation of oxygen-18. FEBS Letters 50:288-290.

5. Baggi, G., D. Catelani, E. Galli, and V. Treccani. 1972. The microbial degradation of phenylalkanes: 2-phenylbutane, 3-phenylpentane, 3-phenyldodecane and 4-phenylheptane. Biochem. J. 126:1091-1097.

6. Bayly, R. C., and G. J. Wigmore. 1973. Metabolism of phenol and cresols by mutants of *Pseudomonas putida*. J. Bacteriol. 113:1112-1120.

7. Basso, L. V., D. R. Rao, and V. W. Rodwell. 1962. Metabolism of pipecolic acid in a *Pseudomonas* species. II. Δ'-Piperideine-6-carboxylic acid and α-aminoadipic acid-δ-semialdehyde. J. Biol. Chem. 237:2239-2245.

8. Beam, H. W., and J. J. Perry. 1974. Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalism. J. Gen. Microbiol. 82:163-169.

9. Behrman, E. J., and R. Y. Stanier. 1957. The bacterial oxidation of nicotinic acid. J. Biol. Chem. 228:923-945.

10. Blakley, E. R. 1974. The microbial degradation of cyclohexanecarboxylic acid: a pathway involving aromatization to form *p*-hydroxybenzoic acid. Can. J. Microbiol. 20:1297-1306.

11. Bourquin, A. W., and V. A. Przybyszewski. 1977. Distribution of bacteria with nitrilotriacetate-degrading potential in an estuarine environment. Appl. Environ. Microbiol. 34:411-418.

12. Bradshaw, W. H., H. E. Conrad, E. J. Corey, I. C. Gunsalus, and D. Lednicer. 1959. Microbial degradation of (+)-camphor. J. Am. Chem. Soc. 81:5507.

13. Britton, L. N., and A. J. Markovetz. 1977. A novel ketone monooxygenase from *Pseudomonas cepacia*. Purification and properties. J. Biol. Chem. 252:8561-8566.

14. Cain, R. B., and D. R. Farr. 1968. Metabolism of aryl sulphonates by microorganisms. Biochem. J. 106:859-877.

15. Cardini, G., and P. Jurtshuk. 1968. Cytochrome P_{450} involvement in the oxidation of *n*-octane by cell-free extracts of *Corynebacterium* sp. strain 7E1C. J. Biol. Chem. 243:6070-6072.

16. Cartwright, N. J., and R. B. Cain. 1959. Bacterial degradation of the nitrobenzoic acids. Biochem. J. 71:248-261.

17. Catelani, D., A. Colombi, C. Sorlini, and V. Treccani. 1973. Metabolism of biphenyl. 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate: the *meta*-cleavage product from 2,3-dihydroxybiphenyl by *Pseudomonas putida*. Biochem. J. 134:1063-1066.

18. Catelani, D., A. Colombi, C. Sorlini, and V. Treccani. 1977. Metabolism of quaternary carbon compounds: 2,2-dimethylheptane and tertbutylbenzene. Appl. Environ. Microbiol. 34:351-354.

19. Chapman, P. J. 1972. An outline of reaction sequences used for the bacterial degradation of phenolic compounds, p. 17-55. *In* Degradation of synthetic organic molecules in the biosphere. National Academy of Sciences, Washington, D.C.

20. Chapman, P. J. 1976. Microbial degradation of halogenated compounds. Biochem. Soc. Trans. 4:463-466.

21. Chapman, P. J., and S. Dagley. 1962. Oxidation of homogentisic acid by cell-free extracts of a vibrio. J. Gen. Microbiol. 28:251-256.

22. Chapman, P. J., and R. G. Duggleby. 1967. Dicarboxylic acid catabolism by bacteria. Biochem. J. 103:7C.

23. Chapman, P. J., G. Meerman, and I. C. Gunsalus. 1965. The microbiological transformation of fenchone. Biochem. Biophys. Res. Commun. 20:104-108.

24. Chapman, P. J., G. Meerman, I. C. Gunsalus, R. Srinivasan, and K. L. Rhinehart. 1966. A new acyclic acid metabolite in camphor oxidation. J. Am. Chem. Soc. 88:618-619.

25. Chapman, P. J., and D. W. Ribbons. 1976. Metabolism of resorcinylic compounds by bacteria: alternative pathways for resorcinol catabolism in *Pseudomonas putida*. J. Bacteriol. 125:985-998.

26. Chouteau, J., E. Azoulay, and J. C. Senez. 1962. Anaerobic formation of n-hept-1-ene from n-heptane by resting cells of *Pseudomonas aeruginosa*. Nature 194:576-578.

27. Colby, J., and H. Dalton. 1976. Some properties of a soluble methane monooxygenase from *Methylococcus capsulatus* strain Bath. Biochem. J. 157:495-497.

28. Crawford, R. L. 1975. Novel pathway for degradation of protocatechuic acid in *Bacillus* species. J. Bacteriol. 121:531-536.

29. Crawford, R. L., S. W. Hutton, and P. J. Chapman. 1975. Purification and properties of gentisate 1,2-dioxygenase from *Moraxella osloensis*. J. Bacteriol. 121:794-799.

30. Cripps, R. E. 1973. The microbial metabolism of thiophen-2-carboxylate. Biochem. J. 134:353-366.

31. Dagley, S. 1971. Catabolism of aromatic compounds by microorganisms. Adv. Microb. Physiol. 6:1-46.

32. Dagley, S., P. J. Chapman, D. T. Gibson, and J. M. Wood. 1964. Degradation of the benzene nucleus by bacteria. Nature 202:775-778.

33. Daly, J., and G. Guroff. 1968. Production of *m*-methyltyrosine and *p*-hydroxymethylphenylalanine from *p*-methylphenylalanine by phenylalanine hydroxylase. Arch. Biochem. Biophys. 125:136-141.

34. Daly, J. W., D. M. Jerina, and B. Witkop. 1972. Arene oxides and the NIH shift: the metabolism, toxicity and carcinogenicity of aromatic compounds. Experientia 28:1129-1149.

35. Davis, J. B., and R. L. Raymond. 1961. Oxidation of alkyl-substituted cyclic hydrocarbons by a *Nocardia* during growth on *n*-alkanes. Appl. Microbiol. 9:383-388.

36. Dean-Raymond, D., and R. Bartha. 1975. Biodegradation of some polynuclear aromatic petroleum components by marine bacteria. Dev. Ind. Microbiol. 16:97-110.

37. Dodson, R. M., and R. D. Muir. 1961. Microbiological transformations. VI. The microbiological aromatization of steroids. J. Am. Chem. Soc. 83:4627-4631.

38. Donoghue, N. A., D. B. Norris, and P. W. Trudgill. 1976. Purification and properties of cyclohexanone oxygenase from *Nocardia globerula* CL-1 and an *Acinetobacter* NCIB 9871. Eur. J. Biochem. 63:175-192.

39. Donoghue, N. A., and P. W. Trudgill. 1975. The metabolism of cyclohexanol by *Acinetobacter* NCIB 9871. Eur. J. Biochem. 60:1-7.

40. Dutton, P. L., and W. C. Evans. 1969. The metabolism of aromatic compounds by *Rhodopseudomonas palustris*. Biochem. J. 113:525-536.

41. Engelhardt, G., P. R. Wallnöfer, and R. Plapp. 1973. Purification and properties of an aryl acylamidase of *Bacillus sphaericus*, catalyzing the hydrolysis of various phenylamide herbicides and fungicides. Appl. Microbiol. 26:709-718.

42. Evans, W. C. 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature 270:17-22.

43. Evans, W. C., B. S. W. Smith, P. Moss, and H. N. Fernley. 1971. Bacterial metabolism of 4-chlorophenoxyacetate. Biochem. J. 122:509-517.

44. Evans, W. C., B. S. W. Smith, H. N. Fernley, and J. I. Davies. 1971. Bacterial metabolism of 2,4-dichlorophenoxyacetate. Biochem. J. 122:543-551.

45. Faulkner, J. K., and D. Woodcock. 1967. Fungal detoxication. VII. Metabolism of 2,4-dichlorophenoxyacetic and 4-chloro-2-methylphenoxyacetic acids by *Aspergillus niger*. J. Chem. Soc., p. 1187-1191.

46. Flashner, M. S., and V. Massey. 1974. Flavoprotein oxygenases, p. 245-283. *In* O. Hayaishi (ed.), Molecular mechanisms of oxygen activation. Academic Press, New York.

47. Focht, D. D., and M. Alexander. 1970. Bacterial degradation of diphenylmethane, a DDT model substrate. Appl. Microbiol. 20:608-611.

48. Focht, D. D., and H. Joseph. 1974. Degradation of 1,1-diphenylethylene by mixed cultures. Can. J. Microbiol. 20:631-635.

49. Francis, A. J., R. J. Spanggord, G. I. Ouchi, R. Bramhall, and N. Bohonos. 1976. Metabolism of DDT analogues by a *Pseudomonas* sp. Appl. Environ. Microbiol. 32:213-216.

50. Frank, L., and B. Ranhand. 1964. Proline metabolism in *Escherichia coli*. III. The proline catabolic pathway. Arch. Biochem. Biophys. 107:325-331.

51. Fried, J., W. Thoma, and A. Klingsberg. 1953. Oxidation of steroids by microorganisms. III. Side chain degradation, ring D-cleavage and dehydrogenation in ring A. J. Am. Chem. Soc. 75:5764-5765.

52. Friello, D. A., J. R. Mylroie, D. T. Gibson, J. E. Rogers, and A. M. Chakrabarty. 1976. XYL, a non conjugative xylene-degradative plasmid in *Pseudomonas* pxy. J. Bacteriol. 127:1217-1224.

53. Fujioka, M., and H. Wada. 1968. The bacterial oxidation of indole. Biochim. Biophys. Acta 158:70-78.

54. Fujiwara, M., L. A. Golovleva, Y. Saeki, M. Nozaki, and O. Hayaishi. 1975. Extradiol cleavage of 3-substituted catechols by an intradiol dioxygenase, pyrocatechase, from a *Pseudomonad*. J. Biol. Chem. 250:4848-4855.

55. Gamar, Y., and J. K. Gaunt. 1971. Bacterial metabolism of 4-chloro-2-methylphenoxyacetate. Formation of glyoxylate by side-chain cleavage. Biochem. J. 122:527-531.

56. Gibson, D. T. 1971. The microbial oxidation of aromatic hydrocarbons. Crit. Rev. Microbiol. 1:199-223.

57. Gibson, D. T. 1968. Microbial degradation of aromatic compounds. Science 161:1093-1097.

58. Gibson, D. T., B. Gschwendt, W. K. Yeh, and V. M. Kobal. 1973. Initial reactions in the oxidation of ethylbenzene by *Pseudomonas putida*. Biochemistry 12:1520-1528.

59. Gibson, D. T., K. C. Wang, C. J. Sih, and H. Whitlock. 1966. Mechanisms of steroid oxidation by microorganisms. IX. On the mechanism of ring A cleavage in the degradation of 9,10-*seco* steroids by microorganisms. J. Biol. Chem. 241:551-559.

60. Goldman, P. 1972. Enzymology of carbon-halogen bonds, p. 147-165. *In* Degradation of synthetic organic molecules in the biosphere. National Academy of Sciences, Washington, D.C.

61. Goodman, D. S., and H. S. Huang. 1965. Biosynthesis of vitamin A with rat intestinal enzymes. Science 149:879-880.

62. Griffin, M., and P. W. Trudgill. 1972. The metabolism of cyclopentanol by *Pseudomonas* NCIB 9872. Biochem. J. 129:595-603.

63. Gunsalus, I. C., and V. P. Marshall. 1971. Monoterpene dissimilation: chemical and genetic models. Crit. Rev. Microbiol. 1:291-310.

64. Guroff, G., and C. A. Rhoads. 1967. Phenylalanine hydroxylase from *Pseudomonas* species (ATCC 11299a). Purification of the enzyme and activation by various metal ions. J. Biol. Chem. 242:3641-3645.

65. Habig, W. H., M. J. Pobjst, and W. B. Jakoby. 1974. Glutathione S-transferase. J. Biol. Chem. 249:7130-7139.

66. Hammond, M. W., and M. Alexander. 1972. Effect of chemical structure on microbial degradation of methyl-substituted aliphatic acids. Environ. Sci. Technol. 6:732-735.

67. Hareland, W. A., R. L. Crawford, P. J. Chapman, and S. Dagley. 1975. Metabolic function and properties of 4-hydroxyphenylacetic acid 1-hydroxylase from *Pseudomonas acidovorans*. J. Bacteriol. 121:272-285.

68. Hayaishi, O., and M. Nozaki. 1969. Nature and mechanisms of oxygenases. Science 164:389-396.

69. Healy, J. B., and L. Y. Young. 1978. Catechol and phenol degradation by a methanogenic population of bacteria. Appl. Environ. Microbiol. 35:216-218.

70. Hector, M. L., and R. R. Fall. 1976. Evidence for distinct 3-methylcrotonyl-CoA and geranyl-CoA carboxylases in *Pseudomonas citronellolis*. Biochem. Biophys. Res. Commun. 71:746-753.

71. Hegeman, G. D. 1972. The evoluation of metabolic pathways in bacteria, p. 56-72. *In* Degradation of synthetic organic molecules in the biosphere. National Academy of Sciences, Washington, D.C.

72. Hochstein, L. I., and B. P. Dalton. 1965. The hydroxylation of nicotine: the origin of the hydroxyl oxygen. Biochem. Biophys. Res. Commun. 21:644-648.

73. Hoet, P. P., and R. Y. Stanier. 1970. The dissimilation of higher dicarboxylic acids by *Pseudomonas fluorescens*. Eur. J. Biochem. 13:65-70.

74. Hopper, D. J., and D. G. Taylor. 1977. The purification and properties of *p*-cresol-(acceptor) oxidoreductase (hydroxylating) a flavocytochrome from *Pseudomonas putida*. Biochem. J. 167:155-162.

75. Horvath, R. S. 1970. Co-metabolism of methyl- and chloro-substituted catechols by an *Achromobacter* sp. possessing a new *meta*-cleaving oxygenase. Biochem. J. 119:871-876.

76. Hou, C. T., R. Patel, and M. O. Lillard. 1977. Extradiol cleavage of 3-methylcatechol by catechol 1,2-dioxygenase from various microorganisms. Appl. Environ. Microbiol. 33:725-727.

77. Howard, P. H., J. Saxena, and H. Sikka. 1978. Determining the fate of chemicals. Environ. Sci. Technol. 12:398-407.

78. Hunt, A. L., D. E. Hughes, and J. M. Lowenstein. 1958. The hydroxylation of nicotinic acid by *Pseudomonas fluorescens*. Biochem. J. 69:170-173.

79. Ichihara, A., K. Adachi, K. Hosokawa, and Y. Takeda. 1962. The enzymatic hydroxylation of aromatic carboxylic acids; substrate specificities of anthranilate and benzoate oxidases. J. Biol. Chem. 237:2296-2302.

80. Iizuka, H., M. Iida, and S. Fujita. 1969. Formation of *n*-decene-1-1 from *n*-decane by resting cells of *Candida rugosa*. Z. Allg. Mikrobiol. 9:223-226.

81. Jeffrey, A. M., D. M. Jerina, R. Self, and W. C. Evans. 1972. The bacterial degradation of flavonoids. Oxidative fission of the A-ring of dihydrogossypetin by a *Pseudomonas* sp. Biochem. J. 130:383-390.

82. Jeffrey, A. M., M. Knight, and W. C. Evans. 1972. The bacterial degradation of flavonoids. Hydroxylation of the A-ring of taxifolin by a soil pseudomonad. Biochem. J. 130:373-381.

83. Jones, N., and G. K. Watson. 1976. Ethylene glycol and polyethylene glycol catabolism by a sewage bacterium. Biochem. Soc. Trans. 4:891-892.

84. Jurtshuk, P., and G. E. Cardini. 1971. The mechanism of hydrocarbon oxidation by a *Corynebacterium* species. Crit. Rev. Microbiol. 1:239-289.

85. Kaneda, T. 1974. Enzymatic aromatization of 4-ketocyclohexane carboxylic acid to p-hydroxybenzoic acid. Biochem. Biophys. Res. Commun. 58:140-144.

86. Kaufman, D. D., and J. Blake. 1973. Microbial degradation of several acetamide, acylanilide, carbamate toluidine, and urea pesticides. Soil Biol. Biochem. 5:297-308.

87. Kawai, F., T. Kimura, M. Fukaya, Y. Tani, K. Ogata, T. Ueno, and H. Fukami. 1978. Bacterial oxidation of polyethylene glycol. Appl. Environ. Microbiol. 35:679-684.

88. Keenan, S. L., and P. J. Chapman. 1978. Carboxyl migration facilitated by bacterial hydroxylation of 4-hydroxybenzoate. Chem. Commun. (in press).

89. Kido, T., K. Soda, T. Suzuki, and K. Asada. 1976. A new oxygenase, 2-nitropropane dioxygenase of *Hansenula mrakii*. Enzymologic and spectrophotometric properties. J. Biol. Chem. 251:6994-7000.

90. Kieslich, K. 1976. Microbial transformations of non-steroid cyclic compounds. Georg Thieme Pub., Stuttgart.

91. Kiyohara, H., and K. Nagao. 1978. The catabolism of phenanthrene and naphthalene by bacteria. J. Gen. Microbiol. 105:69-75.

92. Klug, M. J., and A. J. Markovetz. 1968. Degradation of hydrocarbons by members of the genus *Candida*. III. Oxidative intermediates from 1-hexadecene and 1-heptadecene by *Candida lipolytica*. J. Bacteriol. 96:1115-1123.

93. Knackmuss, H.-J., W. Beckmann, E. Dorn, and W. Reineke. 1976. On the mechanism of the biological persistence of halogenated and sulfonated aromatic hydrocarbons. Zentralbl. Bakteriol. Hyg. B162:127-137.

94. Kodama, K., K. Umehara, K. Shimizu, S. Nakatani, Y. Miroda, and K. Yamada. 1973. Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. Agric. Biol. Chem. 37:45-50.

95. Krishnamurty, H. G., and F. J. Simpson. 1970. Degradation of rutin by *Aspergillus flavus*. Studies with oxygen 18 on the action of a dioxygenase on quercetin. J. Biol. Chem. 245:1467-1471.

96. Kusunose, M., E. Kusunose, and M. J. Coon. 1964. Enzymatic ω-oxidation of fatty acids. I. Products of octanoate, decanoate and laurate oxidation. J. Biol. Chem. 239:1374-1380.

97. Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinckia* species. Appl. Environ. Microbiol. 34:783-790.

98. Lanzilotta, R. P., D. G. Bradley, and M. L. Maddox. 1977. Stereospecific sulfur oxidation of 7-methylthioxanthone-2-carboxylic acid by *Calonectria decora*. Appl. Environ. Microbiol. 34:56-59.

99. Larway, P., and W. C. Evans. 1965. Metabolism of quinol and resorcinol by soil pseudomonads. Biochem. J. 95:52P.

100. Laskin, A. I., P. Grabowich, C. D. Meyers, and J. Fried. 1964. Transformations of eburicoic acid. V. Cleavage of ring A by the fungus *Glomerella fusarioides*. J. Med. Chem. 7:406-409.

101. Lebeault, J. M., B. Roche, Z. Duvnjak, and E. Azoulay. 1969. General scheme for the metabolisation of hydrocarbons by *Candida tropicalis*. Antonie van Leeuwenhoek J. Serol. Microbiol. 35:F34-F34.

102. Lee, Y.-L. T., V. L. Sparnins, and S. Dagley. 1978. Catabolism of 2,4,5-trimethoxybenzoic acid and 3-methoxy-crotonic acid. Appl. Environ. Microbiol. 35:817-819.

103. Magee, P. T., and E. E. Snell. 1966. The bacterial degradation of pantothenic acid. IV. Enzymatic conversion of aldopantoate to α-ketoisovalerate. Biochemistry 5:409-416.

104. Matsuura, T., H. Matsushima, and H. Sakamoto. 1967. Photosensitized oxygenation of 3-hydroxyflavones. A possible model for biological oxygenation. J. Am. Chem. Soc. 89:6370-6371.

105. Markovetz, A. J. 1971. Subterminal oxidation of aliphatic hydrocarbons by microorganisms. Crit. Rev. Microbiol. 1:225-237.

106. Massey, L. K., J. R. Sokatch, and R. S. Conrad. 1976. Branched-chain amino acid catabolism in bacteria. Bacteriol. Rev. 40:42-54.

107. May, S. W., and B. J. Abbott. 1973. Enzymatic epoxidation. II. Comparison between the epoxidation and hydroxylation reactions catalyzed by the ω-hydroxylation system of *Pseudomonas oleovorans*. J. Biol. Chem. 248:1725-1730.

108. McKenna, E. J. 1972. Microbial metabolism of *normal* and branched chain alkanes, p. 73-97. *In* Degradation of synthetic organic molecules in the biosphere. National Academy of Sciences, Washington, D.C.

109. McKenna, E. J., and R. E. Kallio. 1972. Microbial metabolism of the isoprenoid alkane pristane. Proc. Nat. Acad. Sci. U.S.A. 68:1552-1554.

110. Oesch, F. 1973. Mammalian epoxide hydrases. Inducible enzymes catalyzing the inactivation of carcinogenic and cytotoxic metabolites derived from aromatic and olefinic compounds. Xenobiotica 3:305-340.

111. Ogasawara, N., J. E. Gander, and L. M. Henderson. 1965. Purification and properties of 3-hydroxyanthranilate oxygenase from beef kidney. J. Biol. Chem. 241:613-619.

112. Olson, J. A., and O. Hayaishi. 1965. The enzymatic cleavage of β-carotene into vitamin A by soluble enzymes of rat liver and intestine. Proc. Soc. Nat. Acad. Sci. 54:1364-1370.

113. Ornston, M. K., and L. N. Ornston. 1972. The regulation of the β-ketoadipate pathway in *Pseudomonas acidovorans* and *Pseudomonas testosteroni*. J. Gen. Microbiol. 73:455-464.

114. Parekh, V. R., R. W. Traxler, and J. M. Sobek. 1977. n-Alkane oxidation enzymes of a pseudomonad. Appl. Environ. Microbiol. 33:881-884.

115. Perry, J. J. 1977. Microbial metabolism of cyclic hydrocarbons and related compounds. Crit. Rev. Microbiol. 5:387-412.

116. Peterson, D., and J. Llaneza. 1974. Identification of a carbon-oxygen lyase activity cleaving the ether linkage in carboxymethyloxysuccinic acid. Arch. Biochem. Biophys. 162:135-146.

117. Peterson, D. H., S. H. Eppstein, P. D. Meister, H. C. Murray, H. M. Leigh, A. Weintraub, and L. M. Reineke. 1953. Microbiological transformations of steroids. IX. Degradation of C_{21} steroids to C_{19} ketones and to testololactone. J. Am. Chem. Soc. 75:5768-5769.

118. Peterson, J. A., D. Basu, and M. J. Coon. 1966. Enzymatic ω-oxidation. I. Electron carriers in fatty acid and hydrocarbon hydroxylation. J. Biol. Chem. 241:5162-5164.

119. Pirnik, M. P. 1977. Microbial oxidation of methylbranched alkanes. Crit. Rev. Microbiol. 5:413-422.

120. Pirnik, M. P., R. M. Atlas, and R. Bartha. 1974. Hydrocarbon metabolism by *Brevibacterium erythrogenes*: normal and branched alkanes. J. Bacteriol. 119:868-878.

121. Porter, D. J. T., J. G. Voet, and H. J. Bright. 1972. Nitromethane: a novel substrate for D-amino acid oxidase. J. Biol. Chem. 247:1951-1953.

122. Prairie, R. L., and P. Talalay. 1963. Enzymatic formation of testololactone. Biochemistry 2:203-207.

123. Preiss, I., and G. Ashwell. 1962. Alginic acid metabolism in bacteria. I. Enzymatic formation of unsaturated oligosaccharides and 4-deoxy-L-*erythro*-5-hexoseulose uronic acid. J. Biol. Chem. 237:309-316.

124. Reiner, A. M. 1971. Metabolism of benzoic acid by bacteria: 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid as an intermediate in the formation of catechol. J. Bacteriol. 108:89-94.

125. Reiner, A. M., and G. D. Hegeman. 1971. Metabolism of benzoic acid by bacteria. Accumulation of (-)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid by a mutant strain of *Alcaligenes eutrophus*. Biochemistry 10:2530-2536.

126. Rho, E. M., and W. C. Evans. 1975. The aerobic metabolism of cyclohexane carboxylic acid by *Acinetobacter anitratum*. Biochem. J. 148:11-15.

127. Ribbons, D. W. 1970. Specificity of monohydric phenol oxidations by *meta* cleavage pathways in *Pseudomonas aeruginosa* T_1. Archiv. Mikrobiol. 74:103-115.

128. Ribbons, D. W. 1972. Metabolism of methoxy- and methylenedioxyphenyl compounds by bacteria, p. 98-115. *In* Degradation of synthetic organic molecules in the biosphere. National Academy of Sciences, Washington, D.C.

129. Ribbons, D. W. 1975. Oxidation of C-1 compounds by particulate fractions from *Methylococcus capsulatus*: distribution and properties of methane-dependent reduced nicotinamide adenine dinucleotide oxidase (methane hydroxylase). J. Bacteriol. 122:1351-1363.

130. Schultz, E., F. E. Engle, and J. M. Wood. 1974. New oxygenases in the degradation of flavones and flavanones. Biochemistry 13:1768-1776.

131. Seubert, W., and E. Fass. 1964. Untersuchungen über den bakteriellen Abbau von Isoprenoiden. V. Der mechanisms des isoprenoidabbaues. Biochem. Z. 341:35-44.

132. Seubert, W., and U. Remberger. 1963. Untersuchungen über den bakteriellen Abbau con Isoprenoiden. II. Die Rolle der Kohlensaure. Biochem. Z. 338:245-264.

133. Siuda, J. F., and J. F. DeBernardis. 1973. Naturally occurring halogenated organic compounds. Lloydia 36:107-143.

134. Smith, D. I., and A. G. Callely. 1975. The microbial degradation of cyclohexane carboxylic acid. J. Gen. Microbiol. 91:210-212.

135. Sorlini, C., and V. Treccani. 1969. Microbial degradation of aliphatic branched compounds: isobutyric, 2,2-dimethylmalonic and 2,2-dimethylsuccinic acids. Experientia 25:1032.

136. Sparnins, V. L., P. J. Chapman, and S. Dagley. 1974. Bacterial degradation of 4-hydroxyphenylacetic acid and homoprotocatechuic acid. J. Bacteriol. 120:159-167.

137. Sparnins, V. L., and S. Dagley. 1975. Alternative routes of aromatic catabolism in *Pseudomonas acidovorans* and *Pseudomonas putida*: gallic acid as a substrate and inhibitor of dioxygenases. J. Bacteriol. 124:1374-1381.

138. Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43:159-271.

139. Stewart, J. E., W. R. Finnerty, R. E. Kallio, and D. P. Stevenson. 1960. Esters from bacterial oxidation of olefins. Science 132:1254-1255.

140. Stirling, L. A., R. J. Watkinson, and I. J. Higgins. 1977. Microbial metabolism of alicyclic hydrocarbons: isolation and properties of a cyclohexane-degrading bacterium. J. Gen. Microbiol. 99:119-125.

141. Tack, B. F., P. J. Chapman, and S. Dagley. 1972. Metabolism of gallic acid and syringic acid by *Pseudomonas putida*. J. Biol. Chem. 247:6438-6443.

142. Taylor, D. G., and P. W. Trudgill. 1978. Metabolism of cyclohexane carboxylic acid by *Alcaligenes* strain W1. J. Bacteriol. 134:401-411.

143. Tiedje, J. M., J. M. Duxbury, and M. Alexander. 1969. 2,4-D-metabolism: pathway of degradation of chlorocatechols by *Arthrobacter* sp. J. Agr. Food Chem. 17:1021-1026.

144. Toms, A., and J. M. Wood. 1970. The degradation of *trans*-ferulic acid by *Pseudomonas acidovorans*. Biochemistry 9:337-343.

145. Toms, A., and J. M. Wood. 1970. Early intermediates in the degradation of α-conidendrin by a *Pseudomonas multivorans*. Biochemistry 9:733-740.

146. Trudgill, P. W. 1969. The metabolism of 2-furoic acid by *Pseudomonas* F_2. Biochem. J. 113:577-587.

147. Tweedy, B. G., C. Loeppky, and J. A. Ross. 1970. Metabolism of 3-(p-bromo-phenyl)-1-methoxy-1-methylurea by selected soil microorganisms. J. Agric. Food Chem. 18:851-853.

148. Wagner, F., W. Zahn, and U. Buhring. 1967. 1-Hexadecene, an intermediate in the microbial oxidation of n-hexadecane in vivo and in vitro. Angew. Chem. Internat. Ed. 6:359-360.

149. Ward, D. M., and T. D. Brock. 1978. Hydrocarbon biodegradation in hypersaline environments. Appl. Environ. Microbiol. 35:353-359.

150. Watson, G. K., and R. B. Cain. 1975. Microbial metabolism of the pyridine ring. Metabolic pathways of pyridine biodegradation by soil bacteria. Biochem. J. 146:157-172.

151. Willetts, A. J. 1973. Microbial metabolism of alkylbenzene sulphonates. Fungal metabolism of 1-phenylundecane-p-sulphonate and 1-phenyldodecane-p-sulphonate. Antonie van Leeuwenhoek J. Microbiol. Serol. 38:585-597.

152. Willetts, A. J., and R. B. Cain. 1972. Microbial metabolism of alkylbenzene sulphonates. Bacterial metabolism of undecylbenzene-p-sulphonate and dodecylbenzene-p-sulphonate. Biochem. J. 129:389-402.

153. Williams, P. A., and M. J. Worsey. 1976. Ubiquity of plasmids in coding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL plasmids. J. Bacteriol. 125:818-828.

154. Wright, K. A., and R. B. Cain. 1972. Microbial metabolism of pyridinium compounds. Metabolism of 4-carboxy-1-methylpyridinium chloride, a protolytic product of Paraquat. Biochem. J. 128:543-559.

155. Yamada, Y., H. Motoi, S. Kinoshita, N. Takada, and H. Okada. 1975. Oxidative degradation of squalene by *Arthrobacter* sp. Appl. Microbiol. 29:400-404.

156. Yang, H.-C., M. Kusumoto, S. Iwahara, T. Tochikura, and K. Ogata. 1969. Studies on degradation of *d*-biotin by microorganisms. I. *d*-Biotin degradation by yeasts and molds. Agric. Biol. Chem. 33:1730-1736.

ROLE OF COMETABOLISM

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ABSTRACT

Cometabolism is the metabolism by a microbial population of a chemical which that species cannot use as a nutrient or an energy source. The cometabolizing population does not increase in abundance in response to introduction of that chemical into natural ecosystems. Because the rate of transformation of many chemicals parallels the rate of population growth, a common consequence of cometabolism is a slow microbial transformation. Furthermore, products typically accumulate in a cometabolic process, the accumulated metabolite being structurally similar to the original compound. Examples of cometabolism are found among pesticides, chemicals used in the home, and products of importance to the manufacturing industry. A probable explanation of cometabolism is the existence in the active population of an enzyme of broad substrate specificity, the initial enzymatic reaction yielding products that do not serve as substrates for other enzymes present in the responsible cells. The possible role of cometabolism in nature is discussed.

Microbiologists and biochemists have traditionally limited their attention in investigations of catabolism to a small number of compounds. Although it is widely believed that the number is large, the range includes but a few sugars, amino acids and nucleotides and their polymers, fatty acids and a modest assortment of simple aliphatic and aromatic hydrocarbons. These studies have resulted in an enormous and valuable literature, but much of the research has little relevance to modern environmental problems. The reason is that the types of molecules of current environmental importance, and of concern to regulatory agencies, include several hundred pesticides as well as tens of thousands of chemicals of industrial importance, few of which are structurally similar to the common laboratory substrates. The reason for the attention to the number of nonpesticidal compounds is the enactment of the Toxic Substances Control Act, which brings under regulatory view an array of molecules heretofore not considered by biologists or environmental scientists.

Environmental monitoring has clearly discredited the view that all organic molecules are biodegradable, and this fanciful notion was brought to a sudden demise under the weight of the many new chemicals of potential environmental significance. Furthermore, the generally held view that all molecules which microorganisms can metabolize will also provide them with a source of carbon and energy has also fallen beneath the weight of new chemicals.

Compounds in the ever-increasing assortment that are of potential regulatory concern can be divided into three broad groups. First are those that can be used as a source of carbon, some other nutrient element or energy. When these compounds are introduced into a natural ecosystem, the heterotrophs able to grow on them proliferate, and the small initial population active on these substrates increases in size with time. Parallel to increase in numbers or biomass of active cells is an ever-increasing rate of destruction of the molecule. Essentially all of the chemical will disappear in reasonably short periods of time, and the products are either carbon dioxide or molecules unrelated to the parent substance. Second are those chemicals that are totally recalcitrant. These are not transformed microbiologically because no enzymes can modify them or because they do not penetrate into microbial cells that contain the requisite enzymes. These molecules thus frequently persist, unless there is some nonbiological mechanism for their modification or downward movement.

The third group of compounds includes those subject to cometabolism. Cometabolism refers to the metabolism by a microorganism of a chemical which that organism cannot use as a nutrient or an energy source. Although there is some confusion and overlap with the term "cooxidation," the former term is more desirable because many of the processes involved do not entail an oxidation.

Although most investigations of cometabolism have centered on compounds that do not contain nutrient elements other than carbon, hydrogen and oxygen, the substrate acted on by the cometabolizing population also might not serve as a source of any nutrient which may be limiting in a particular environment; thus, the organism may not use the molecule as a source of nitrogen, sulfur or phosphorus, if these elements exist in the structure.

Several outcomes of cometabolism are almost self-evident. For example, the compound that serves as a nutrient source provides the active populations with a selective advantage, and they become more abundant in time. However, a chemical that does not serve as a nutrient does not give the organism acting on it a selective advantage; hence, its cell density or biomass does not rise with time. This lack of population growth during cometabolism has rarely been shown in nature or even in culture, but it would seem to be a natural consequence of the phenomenon. Furthermore, inasmuch as biochemical activity in a natural ecosystem is roughly proportional to the number or biomass of cells carrying out the specific transformation, the absence of an increase in population size would be reflected in the lack of a rapid rise in the rate of biodegradation, as is typical of those substrates that support microbial growth.

As an example, consider a synthetic organic compound that no population in a particular environment can use as a carbon source. Inasmuch as growth on organic molecules requires their conversion to precursors for biosynthesis of amino acids, sugars, nucleic acids and fatty acids incorporated into cell constituents, organisms failing to grow on the compounds in question must be unable to form these intermediates. Were they able to do so, the populations would proliferate. If precursors required for biosynthetic purposes are not generated, a product that the organism forms from the parent molecule would likely accumulate. This would be the end of the metabolic sequence in transformation of the synthetic molecule. Hence, a consequence of cometabolism is the formation of one or more products that are structurally related to the original substrate, rather than the typical products of complete degradation. Such accumulations have been observed in cultures of many bacteria and fungi provided with a wide range of organic compounds.

In some of our studies, for example, DDT and its analogues were found to be transformed in cell suspensions or samples of water into a series of products similar to those reported by other investigators. On the other hand, it was observed that one of the two rings of the insecticide molecule could be cleaved by a cometabolizing population to give a product with a single benzene ring. This compound, however, would not sustain growth of the original cometabolizimg population (5, 6, 14, 15, 17). In other of our studies, it was observed that nitro aromatic compounds (16), certain phenoxy pesticides (11), and benzoates (8, 9) were cometabolized by individual cultures, and in the process, were converted to products quite similar in structure to the original molecule (Fig. 1, 2, 3). Our studies in this connection are not unique, and many other microorganisms have been observed to cometabolize a multitude of chemicals of dissimilar structures to yield products that the active species could not further degrade (3, 4, 7, 10, 12, 13, 21, 22). Chemicals acted on in this manner include insecticides, herbicides, surfactants, aliphatic and aromatic hydrocarbons, as well as chemicals that are widely used in industry as solvents or precursors for manufactured products. The reactions involve dehalogenation, introduction of hydroxyl groups, ring cleavage, epoxidation and oxidation of methyl groups to the corresponding alcohols or aldehydes. Hence, many microorganisms have the capacity for cometabolism, the rangeof substrates is truly impressive and a variety of biochemical reactions can be effected by the responsible heterotrophs.

How does one explain cometabolism? For the teleologist, it is comforting to believe that every enzymatic reaction that microorganisms catalyze does the organism some good, but this is obviously sheer teleology and has no basis in fact. Many enzymatically catalyzed conversions are fortuitous, and the organism obtains no value from the reaction that it effects. It is our view that the likeliest explanation is simply that the molecule undergoing cometabolism is acted on by an enzyme that normally serves a physiological function; however, the products of the reaction with the substrate for cometabolism are not further transformed by enzymes of the active populations. Thus, should the initial enzyme acting on a physiological substrate have a broad specificity, it may transform not only the normal substrate but related molecules. In the former instance, the product of the reaction will be further utilized, but in the latter situation, the product may not be transformed further because the succeeding enzyme has a more narrow substrate specificity and is unable to transform the product made available to it. Some evidence exists to support this view; for example, the enzyme cleaving 2,4-D acts on a number of related phenoxy compounds to yield the corresponding phenols, but the enzymes further degrading the phenols by the widely understood routes of aromatic metabolism act on some, but not all, of the phenols that are generated (2, 11). Those that are not substrates for the phenol hydroxylating enzymes will accumulate, and thus the preceding phenoxy compound will be cometabolized and not support growth.

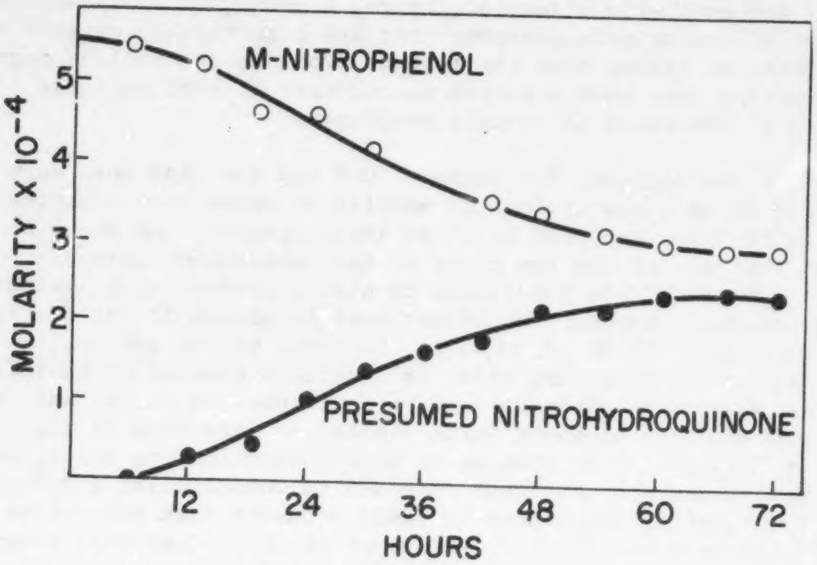

Figure 1. Bacterial conversion of m-nitrophenol to a product absorbing at 540 nm. The product was subsequently identified as nitrohydroquinone.

It should not be surprising to anyone familiar with biochemistry that enzymes are not absolutely specific for their substrates. Many of these catalysts act on a series of closely related molecules. This modest range of substrate specificity is true of simple organic molecules such as organic acids (19) as well as more complex aromatic compounds (18).

It is possible, nevertheless, that cometabolism sometimes is a consequence of the toxicity of an early product in catabolism. The inhibitor, should it not be destroyed essentially as rapidly as it is formed, may build up to levels that prevent microbial replication. The evidence in support of this hypothesis is skimpy, but one might cite the work of Tranter and Cain (20) who suggested that the failure of many fluoro substituted aromatic molecules to support bacterial proliferation might result from the conversion of the compounds to a metabolic inhibitor such as fluoroacetate.

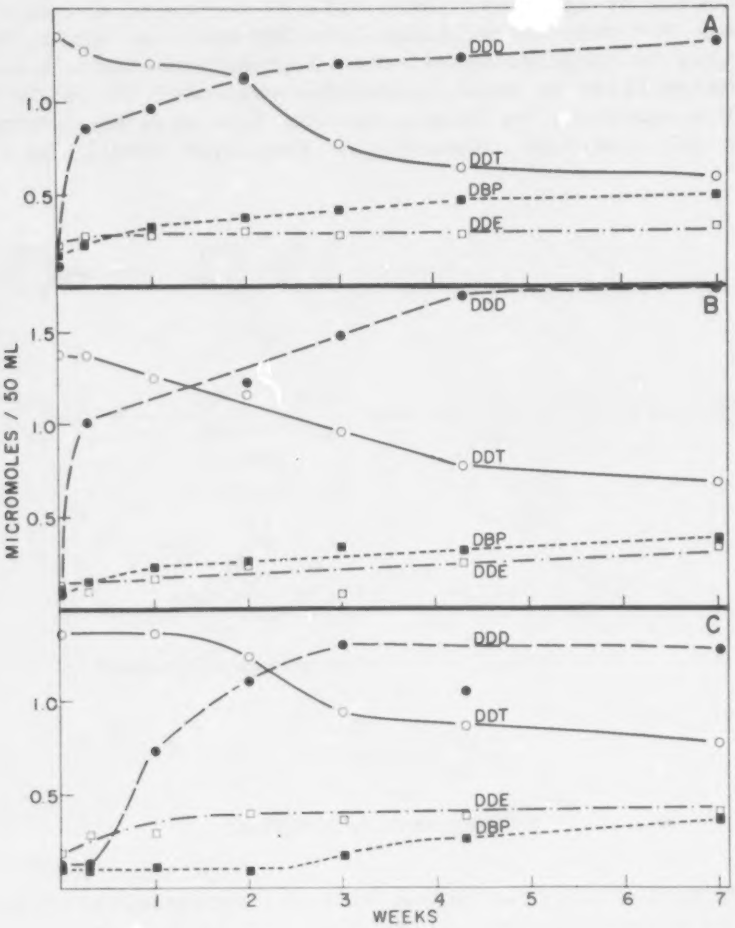

Figure 2. Conversion of DDT to DDD, DDE and DBP in waste water. The three products are all chlorine-containing diphenylmethane derivatives. The samples received (A) no addition, (B) glucose or (C) nonchlorinated diphenylmethane.

As one moves from the purity of the axenic culture to the marine environment, obvious shortcomings of the in vitro view of cometabolism become evident. Thus, products of cometabolism of one species may be excellent substrates for a second species, and indeed the second species may grow on the product and thus lead to its rapid destruction. Under these circumstances, the product of cometabolism will not reach appreciable or possibly even detectable levels. Thus, we have observed that DDT cometabolism in culture gives rise to p-chlorophenylacetic acid. The latter is not further degraded by the original population, but other organisms have been isolated which can grow on the product of cleavage of one of the two rings of the DDT molecule (14). Still, the first phase of this biphasic process will probably be very slow as a rule because the organisms are not deriving energy from the conversion, although the product of the first phase will be destroyed as quickly as it becomes available. Not only insecticides like DDT but also other chemicals may be subject to this two-population effected degradation; thus, 2,3,6-trichlorobenzoate is cometabolized to yield 3,5-dichlorocatechol (8). The latter compound is itself a substrate for microorganisms that grow on related chlorinated aromatics (1), and thus presumably is destroyed totally by species able to grow on it.

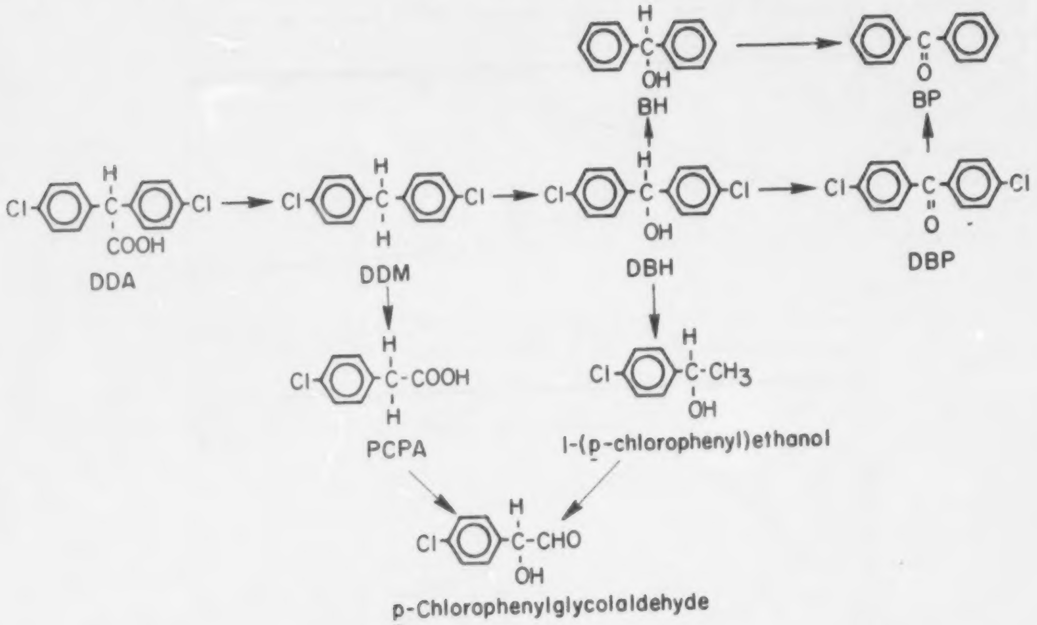

Figure 3. Products and proposed pathway for the cometabolism of analogues of DDT by *Pseudomonas putida*.

What is the evidence that cometabolism actually functions in natural ecosystems? Microbiologists, with their propensity for wishful thinking, assume that anything their pet organisms do in culture, they will also do in nature. Such wishful thinking may in fact reflect processes in natural waters, but meaningful evidence is clearly needed. The best evidence, albeit indirect, comes from studies in soil rather than water. A large number of chemicals, particularly among the pesticides, when added to soil are converted to products similar to the parent molecules. The processes appear to be microbial because the reaction is abolished or markedly reduced in rate upon sterilizing the soil. Inasmuch as the process is the result of microbial processes or is enhanced by microorganisms, the microbiologist endeavors to isolate species carrying out the conversion. In some cases he is successful; in other instances he is not. The failure may be attributable to the absence from the isolation medium of an essential nutrient for the organisms with the correct array of enzymes; thus, most investigators working with synethetic molecules use enrichment media containing the test chemical as the sole carbon source. Yet, heterotrophs that require growth factors will not develop in these media. If growth factors are included in a synthetic medium together with a somewhat resistant organic chemical as the major carbon source, the organisms that typically develop in the enrichment culture are those that use the growth factors as carbon sources rather than synthetic molecule. Granting that the inability to isolate an organism from a natural environment in which a process is occurring may thus frequently result from the absence of growth factors from the isolation medium, it is plausible that often the process in the natural ecosystem arises not from the activities of fastidious populations but rather from cometabolizing ones. A case in point is the herbicide 2,4,5-T, which is converted to the corresponding phenol (Fig. 4); organisms growing on 2,4,5-T are rare or do not exist. In addition, many reasonably resistant organic compounds are degraded more rapidly in samples of natural environments to which are added readily utilizable energy sources, and it is possible that these energy sources are providing carbon and energy for the cometabolizing population so it can transform the subject chemical. These lines of evidence are admittedly not strong, but they are suggestive that cometabolism is responsible for the transformation of many synthetic chemicals.

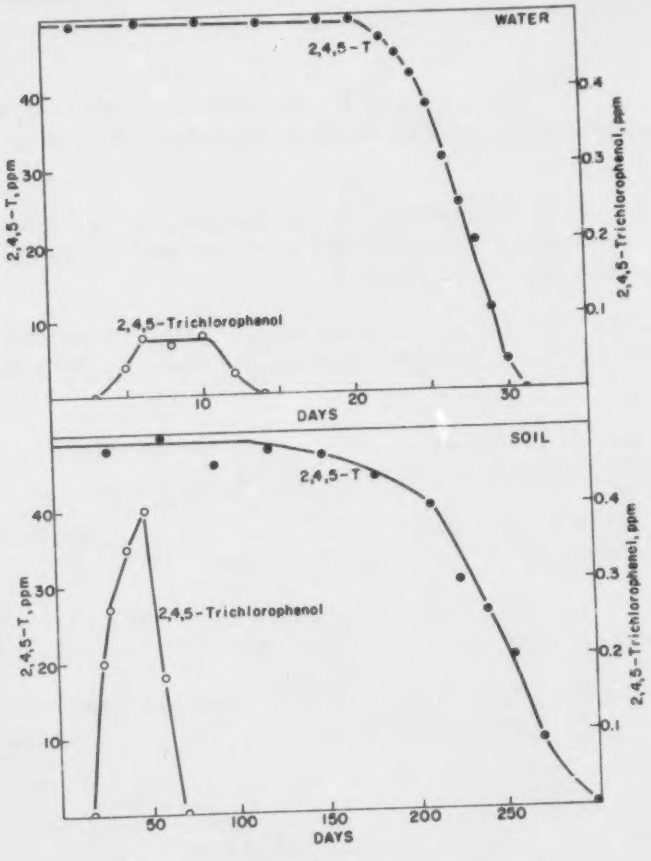

Figure 4. Disappearance of 2,4,5-trichlorophenoxyacetate (2,4,5-T) and formation of the corresponding phenol in samples of freshwater and soil (K. W. Sharpee and M. Alexander, unpublished data).

Many topics for investigation remain for the microbiologist, biochemist and environmental scientist. For example, it is important to establish what chemicals and products are involved in this type of conversion, and which microoorganisms are responsible. Since cometabolic reactions probably are generally slow (unless the responsible populations are initially large and their enzymes are particularly active), then concern with environmental quality dictates that means be sought to increase the rates. Such an enhancement may come about because of an understanding of the process, or it may arise because of a technique discovered in an entirely fortuitous fashion. Finally, because this is a phenomenon that appears to be widespread and of practical significance, information should be obtained on how and why it occurs. With even some information in these various areas of inquiry, it is hoped we shall be in a better position to understand the processes concerned with microbial degradation of pollutants and in maintenance of the quality of marine environments.

LITERATURE CITED

1. Bollag, J.-M., G. G. Briggs, J. E. Dawaon, and M. Alexander. 1968. 2,4-D metabolism. Enzymatic degradation of chlorocatechols. J. Agric. Food Chem. 16:829-833.

2. Bollag, J.-M., C. S. Helling, and M. Alexander. 1968. 2,4-D metabolism. Enzymatic hydroxylation of chlorinated phenols. J. Agric. Food Chem. 16:826-828.

3. Bourquin, A. W., S. K. Alexander, H. K. Speidel, J. E. Mann, and J. F. Fair. 1972. Microbial interactions with cyclodiene pesticides. Develop. Ind. Microbiol. 12:264-276.

4. Flashinski, S. J., and E. P. Lichtenstein. 1974. Degradation of Dyfonate in soil inoculated with *Rhizopus arrhizus*. Can. J. Microbiol. 20: 871-875.

5. Focht, D. D., and M. Alexander. 1970. DDT metabolites and analogs: ring fission by *Hydrogenomonas*. Science 170:91-92.

6. Focht, D. D., and M. Alexander. 1971. Aerobic cometabolism of DDT analogs by *Hydrogenomonas*. J. Agric. Food Chem. 19:20-22.

7. Hashimoto, K. 1973. Oxidation of phenols by yeast. II. Oxidation of cresols by *Candida tropicalis*. J. Gen. Appl. Microbiol. 19:171-187.

8. Horvath, R. S., and M. Alexander. 1970. Cometabolism: a technique for the accumulation of biochemical products. Can. J. Microbiol. 16: 1131-1132.

9. Horvath, R. S., and M. Alexander. 1970. Cometabolism of *m*-chlorobenzoate by an *Arthrobacter*. Appl. Microbiol. 20:254-258.

10. Jensen, H. L. 1963. Carbon nutrition of some microorganisms decomposing halogen-substituted aliphatic acids. Acta Agric. Scand. 13:404-412.

11. Loos, M. A., R. N. Roberts, and M. Alexander. 1967. Phenols as intermediates in the decomposition of phenoxyacetates by an *Arthrobacter* species. Can. J. Microbiol. 13:679-690.

12. Matsumura, F., V. G. Khanvilkar, K. C. Patil, and G. M. Boush. 1971. Metabolism of endrin by certain soil microorganisms. J. Agric. Food Chem. 19:27-31.

13. Patel, R. N., and D. S. Hoare. 1971. Physiological studies of methane and methanol-oxidizing bacteria. J. Bacteriol. 107:187-192.

14. Pfaender, F. K., and M. Alexander. 1972. Extensive microbial degradation of DDT in vitro and DDT metabolism by natural microbial communities. J. Agric. Food Chem. 20:842-846.

15. Pfaender, F. K., and M. Alexander. 1973. Effect of nutrient additions on the apparent cometabolism of DDT. J. Agric. Food Chem. 21:397-399.

16. Raymond, D. G. M., and M. Alexander. 1971. Microbial metabolism and cometabolism of nitrophenols. Pestic. Biochem. Physiol. 1:123-130.

17. Subba-Rao, R. V., and M. Alexander. 1977. Cometabolism of products of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) by *Pseudomonas putida*. J. Agric. Food Chem. 25:855-888.

18. Suggs, J., R. Hawk, A. Curley, E. Boozer, and J. McKinney. 1970. DDT metabolism: oxidation of the metabolite 2,2-bis(*p*-chlorophenyl)ethanol by alcohol dehydrogenase. Science 168:582.

19. Tober, C. L., P. Nicholls, and J. D. Brodie. 1970. Metabolism and enzymology of fluorosuccinic acids. II. Substrate and inhibitor effects with soluble succinate dehydrogenase. Arch. Biochem. Biophys. 138: 506-514.

20. Tranter, E. K., and R. B. Cain. 1967. The degradation of fluoro aromatic compounds to fluorocitrate and fluoroacetate by bacteria. Biochem. J. 103:22P-23P.

21. Walker, J. D., and J. J. Cooney. 1973. Oxidation of *n*-alkanes by *Cladosporium resinae*. Can. J. Microbiol. 19:1325-1330.

22. Willetts, A. J. 1974. Microbial metabolism of alkylbenzene sulfonates. Oxidation of key aromatic compounds by a *Bacillus*. Antonie van Leeuwenhoek 40:547-559.

DIAUXIC AND COMETABOLIC PHENOMENA IN BIODEGRADATION EVALUATIONS

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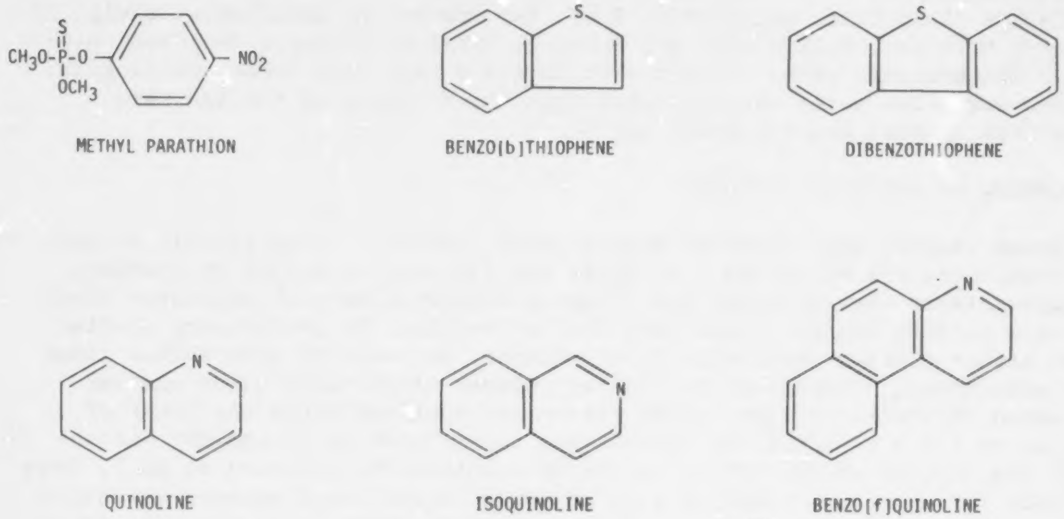
ABSTRACT

Methyl parathion, benzo[b]thiophene, dibenzothiophene, quinoline, benzo[f]quinoline, and isoquinoline are biodegraded by mixed culture systems obtained from organisms in oligotrophic or eutrophic waters, sewage effluents, or polluted estuarine waters. Induction of degradative enzymes and/or cometabolism with structurally-related chemicals is necessary to degrade some of these compounds. The biodegradability of these substances can be affected when nutrients that are assimilated more readily are present in the medium. These facts should be considered in the development of ecosystems to be used for assessing whether pollutants are biodegraded.

INTRODUCTION

Previous papers presented in this workshop described degradative pathways and cometabolism involving pure culture systems. Presently, we are reporting on studies primarily concerned with mixed culture biodegradation during the first stages of relatively large-scale enrichment processes. These latter procedures may be more representative of environmental conditions than pure cultures and small flask processes. We also studied mixed enrichment cultures that were serially propagated on inorganic salts/substrate media, following which commonly used microbial nutrients were added. In this way, we could obtain an insight into the effects of simultaneous pollution by other nutrients. The substrates used were methyl parathion (MP), benzo[b]thiophene (BT), dibenzothiophene (DBT), quinoline (Q), isoquinoline (IQ), and benzo[f]quinoline (BQ). Their structures are presented in Figure 1. Naphthalene (N) and anthracene (A) were used as inducers and/or cometabolic substrates.

In previous studies on the microbial degradation of parathion, both pure cultures (25, 26) and mixed cultures (1, 18) readily degraded the insecticide. The metabolic pathways (17, 19) and the properties of parathion hydrolase (16) have been defined. Several reports also indicated that MP was biodegraded in waters or soils (2, 5, 14, 15, 21, 31). Our prior studies (1, 4, 27) with mixed culture systems suggested that not all organisms degraded MP. In the current study, we have demonstrated that problems may be encountered if other readily assimilable nutrients are present in the media.



METHYL PARATHION BENZO[b]THIOPHENE DIBENZOTHIOPHENE

QUINOLINE ISOQUINOLINE BENZO[f]QUINOLINE

Figure 1. Compounds used for the development of biodegrading cultures by enrichment techniques.

Aerobic degradation of DBT has been investigated extensively (7, 8, 10-13, 29, 30) but most os these studies were conducted with pure cultures. Aerobic degradation of BT was reported less frequently (1, 24, 27-29) and only under mixed substrate conditions. These may have involved cometabolic phenomena but this was not definitely established. Suggestive evidence for biodegradation of Q in soils was obtained in 1916 (23) and 1917 (6). However, before our in-investigations (1, 27) no reports were published on aerobic microbial degradation of IQ or BQ. Q, IQ, and BQ as well as BT and BDT coexist with many types of polycyclic aromatic compounds in coal tars and crude petroleum. Consequently, studies on the biodegradation of these heterocyclic aromatic compounds are of environmental importance.

MATERIALS AND METHODS

CHEMICALS

MP was obtained from Chem Service (West Chester, Pennsylvania); BT (thianaphthene) and DBT were obtained from Aldrich Chemical (Milwaukee, Wisconsin). All were recrystallized from methanol. For enrichment studies, Q (Aldrich) was used without further purification, but was purified by gas chromatography (gc) to produce reference material for analysis. BQ (Eastman, Rochester, New York), IQ (Aldrich), N (Mallinckrodt, St. Louis, Missouri), and A (Sigma, St.

Louis, Missouri) were used without further purification.

WATER SAMPLES

Eutrophic water samples were obtained from two sources: Coyote Creek in San Jose, California, and a pond near Searsville Lake in Woodside, California. Sewage effluents were obtained from treatment plants with different feed characteristics (Palo Alto, California; South San Francisco, California; Shell Oil Refinery, Martinez, California; and Monsanto Chemical Company, Anniston, Alabama). Oligotrophic water samples were obtained from Lake Tahoe, California. Polluted estuarine water was collected from the vicinity of the San Mateo Bridge that crosses San Francisco Bay.

DEVELOPMENT OF ENRICHED CULTURES

Water samples with sediment present were placed in large sterile collection reservoirs for about one h to allow the sediment to settle by gravity. The supernatants were siphoned and filtered through fine-mesh polyester cloth to remove insects and particles that did not settle. In preliminary studies we had higher success rates with large volumes, as compared with shaker flask scale enrichment. Consequently, 4-liter volumes of filtered water samples were added to sterile 9-liter glass fermentors that contained one liter of a solution of 0.5 g of $(NH_4)_2SO_4$ [NH_4NO_3 was substituted in BT and DBT enrichments] and 10 g of KH_2PO_4-K_2HPO_4 buffer to maintain the solution at pH 7. Test chemicals for enrichment studies were added as concentrated aqueous solutions (Q, IQ, BQ hydrochloride, and sterile microbial nutrients as indicated) or as concentrated solutions in dimethyl sulfoxide (MP, BT, DBT, N and A) and mixed thoroughly in the diluted water samples. The initial concentrations of test compounds was 10 µg ml^{-1}. The 9-liter fermentors were fitted for sterile aeration, air exhaust, sampling, and addition of other nutrients as needed. Incubations were carried out at 25°C except for the Lake Tahoe water samples that were maintained at 5-10°C while being transported to the laboratory and then were incubated at 15°C. Cultures were continuously aerated, and samples were taken for analyses at frequencies based on our past experiences with the test compounds. Sampling intervals varied from 2 to 48 h. Cultures were incubated for up to 6 weeks. When BT and/or N were used, they were added to the fermentors at 2-day intervals because control tests indicated that during that time these compounds were removed from the culture media by aeration.

When 50-70% of a test compound was degraded, enrichment cultures were transferred to shaker flasks containing basal inorganic salts media with different concentrations of the test compound. Each liter of basal salts medium contained: 1.4 g of K_2HPO_4, 0.6 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.1 g of NaCl, 0.1 g of $MgSO_4 \cdot 7\ H_2O$, 0.02 g of $CaCl_2 \cdot 2\ H_2O$, 0.005 g of $FeSO_4 \cdot 7\ H_2O$, and 1 ml of trace elements solution. The latter contained 0.1 g of H_3BO_3, 0.05 g each of $CuSO_4 \cdot 5\ H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7\ H_2O$, Na_2MoO_4, and $CoCl_2 \cdot 6\ H_2O$ $liter^{-1}$. In studies in which BT or DBT were substrates, $(NH_4)_2SO_4$ was replaced by NH_4NO_3 and other sulfate salts were replaced with the corresponding chloride salts. Shaker flasks containing BT and N were capped with Teflon-lined screw-on caps.

Separate rotary shaker incubators were used for each individual substrate to prevent contamination of shaker flask media by the vapors of readily metabolized substrates (e.g., N, BT) or by metabolites from transformations.

ANALYSES

Absorption measurements of broths or extracts at critical differential (peak and lower value) uv wavelengths were particularly useful for rapid analyses of the many degradations being conducted. Combinations of wavelengths used were: 270 and 300 nm for MP; 296 and 300 nm for BT; 290 and 300 nm for DBT; 312 and 320 nm for Q; 345 and 355 nm for BQ; and 316 and 324 nm for IQ. Samples from critical points in the degradation curves were always analyzed by gc or high-performance liquid chromatography (hplc).

Samples for analyses were extracted twice with equal volumes of ethyl acetate or hexane. The extracts were adjusted to fixed volumes and dried with Na_2SO_4. Aliquots of the extracts were injected, directly or after being evaporated under nitrogen, into gc or hplc analyzers. MP analyses were made with a Varian Associates Model 5711 with a AFIS detector (Varian Model 2740); the column was 5 ft x 0.25 in. packed with 4% SD-30 and 6% OV-210 on 80/160 mesh Gas Chrom Q; column temperature, 200°C; N_2 carrier gas at 33 ml, min^{-1}; retention time, 3.73 min. Ethyl parathion was used as the internal standard. A Spectra Physics Model 3500 analyzer was used for hplc analyses of heterocyclic hydrocarbons. For BT, a µ Bondapack C_{18} (30 cm x 4 mm) (Waters Associates) column was used with a mobile phase consisting of 60% methanol and 40% water, and a Schoeffel Variable uv detector set at 228 nm (sensitivity, 500 pg). For DBT, the column was Spherisorb ODS (24 cm x 6 mm) (Spectra Physics) with a mobile phase containing 55% methanol and 45% water, and a Spectraphysics 230 uv Detector set at 254 nm (sensitivity, 20 ng). For Q and IQ, the column was µ Bondapack C_{18} (30 cm x 4 mm) (Water Associates) with a mobile phase composed of 60% methanol and 40% water. A Schoeffel Variable uv Dectector was set at 220 nm (sensitivity, 100 pg). For BQ, the column was Spherisorb ODS (24 cm x 5 mm) (Spectraphysics) with a mobile phase consisting of 55% methanol and 45% H_2O. A Schoeffel Variable Fluorescence Detector was used at 360 nm (sensitivity, 4 ng).

RESULTS AND DISCUSSION

METHYL PARATHION (MP)

MP-degrading organisms were present in all eutrophic water bodies and sewage effluents (1, 4, 27). To obtain an adequate supply of cells for determining the kinetic constants defining the degradation of malathion, Paris et al. (22) grew degrading organisms in a medium containing malathion and 1:10 diluted nutrient broth. Because one of our assignments was to obtain rate constants with mixed enrichment cultures from natural water bodies, we also required adequate concentrations of cells. Therefore, we investigated the effect of nutrient broth on a MP-degrading mixed culture system transferred more than 10 times in an inorganic salts/MP medium after the original enrichment degradation. Table 1 shows MP degradations when cultures were grown: (I) in shaker flasks one time in an inorganic salts medium with MP (50 µg ml^{-1}), (II) in nutrient broth (Difco) diluted 1:10 and with MP (50 µg ml^{-1}), and (III) when the mixed culture was transferred daily three times in the diluted nutrient broth/MP medium used in (II) and then the fourth transfer was incubated for 86 h in a similar medium.

TABLE 1. EFFECT OF ORGANIC NUTRIENTS ON METHYL PARATHION (MP) BIODEGRADATION

Medium	Serial transfers (numbers)	Incubation time of final transfer (h)	Degradation (%)
(I) Inorganic salts + MP (50 μg ml^{-1})	1*	21	72
(II) Nutrient broth (0.1 conc.) + MP (50 μg ml^{-1})	1*	21	95
(III) Same as (II)	4**	86	5

*The inoculum used at the initiation of this experiment had already been transferred more than 10 times on inorganic salts/MP medium.

**The first 3 transfers for (III) were made daily.

After one transfer in the diluted nutrient broth/MP medium (II), MP degradation increased slightly in the 21-h incubation compared with the control degradation in inorganic salts/MP medium (I). The nutrient broth supported growth of a greater number of cells. However, when the organisms were transferred four times in the diluted nutrient broth/MP medium (III), MP-degrading capacity was markedly diminished. This was interpreted as being due to ingredients in the nutrient broth that favored growth of organisms other than those involved in degradation, leaving few of the primary MP-degrading organisms and/or exoenzymes involved in modifying the structure of MP.

BENZO[b]THIOPHENE (BT)

Table 2 presents the data from enrichment studies conducted with BT. Because of the volatility of BT, degradations were considered to be positive when transfers made to screw-capped flasks containing inorganic salts medium with BT, or BT plus additive, indicated BT degradation. When BT was the sole substrate, no degradation was observed in six water samples during 6 weeks of incubation. Naphthalene (N) was selected as a cosubstrate because it coexists with BT in coal tars and petroleum products and has a similar chemical structure. BT degradation was observed in four of five water samples to which N was added with BT at 10 μg ml^{-1} levels every 2 days. The one sample that did not develop a BT-degrading culture within 6 weeks under this mixed substrate condition was the oligotrophic sample from Lake Tahoe. When BT, glucose, and yeast extract were added to one water sample in a 9-liter fermentor, BT degradation was not observed. In other studies (27), we found that the breakdown was a cometabolic process in which N could not be replaced by quinoline (Q) using cultures that had been transferred many times on salts/N/BT medium. Similarly, if such a culture mixture was transferred to media in which N was replaced by tryptone or glucose plus yeast extract, BT degradation did not occur (27).

TABLE 2. DEVELOPMENT OF BENZO[b]THIOPHENE(BT)-BIODEGRADING CULTURES IN AQUATIC SYSTEMS

Addition to water samples	Time for development of cultures	BT biodegradation*
BT alone	--†	0/6
BT + naphthalene (N)	5-8 days	4/5
BT + glucose + yeast extract	--†	0/1

*Number of water samples that developed BT-biodegrading cultures/number of different water samples tested.

†Did not develop within 6 weeks of incubation.

DIBENZOTHIOPHENE (DBT)

Naphthalene (N) or anthracene (A) were added to DBT because they coexist with DBT in wastes from the coal and petroleum industries and because they have similar structural characteristics. The selection of these two aromatic hydrocarbons was arbitrary for many other compounds could have been selected. The data in Table 3 indicate that even though DBT-degrading organisms could be obtained by adding DBT as the sole substrate, N was an inducer in development of DBT-degrading organisms. The presence of N caused more rapid degradation of DBT. Although only one enrichment was initiated with the DBT/A combination, the data suggest that A may also be an inducer of enzymes used in DBT degradation. All five mixed cultures developed in the DBT plus N substrate enrichments were able to degrade DBT on subculturing in media with decreasing concentrations of N.

TABLE 3. DEVELOPMENT OF DIBENZOTHIOPHENE(DBT)-BIODEGRADING CULTURES IN AQUATIC SYSTEMS

Additions to water samples	Time for DBT degradation (days)	DBT degradation*
Eutrophic waters and sewage effluents		
DBT alone	17-22	3/4
DBT + naphthalene (N)	2-5	3/5
DBT + anthracene (A)	8	1/1
Oligotrophic waters		
DBT alone	--†	0/1
DBT + N	32	1/1

*Number of water samples that developed DBT-biodegrading cultures/number of different water samples tested.

†Did not develop within 6 weeks.

QUINOLINE (Q)

Cultures that could degrade Q were readily obtained. Figure 2 illustrates the time course of degradations using procedures designated as "Cascade Batch Fermentations" (27). Two concentrations of Q were used. During the first 2 days of incubation, no degradation of Q occurred, following which Q was totally degraded in both cultures. The latter were inoculated (0.2% v/v) into shaker flasks containing freshly collected water samples mixed with one-fifth volumes of $(NH_4)_2SO_4$-potassium phosphate buffer and the respective initial concentrations of Q. These processes were repeated four times. In all instances, short lag periods occurred followed by rapid degradations of Q. These Cascade Batch Fermentations suggest that, under natural conditions, a lag period would occur in which Q would be degraded downstream from the source where it was first introduced, but gradually degradation would occur increasingly closer to the source of pollution. This was demonstrated in a multi-chamber fermentation unit with *p*-cresol as the substrate (3).

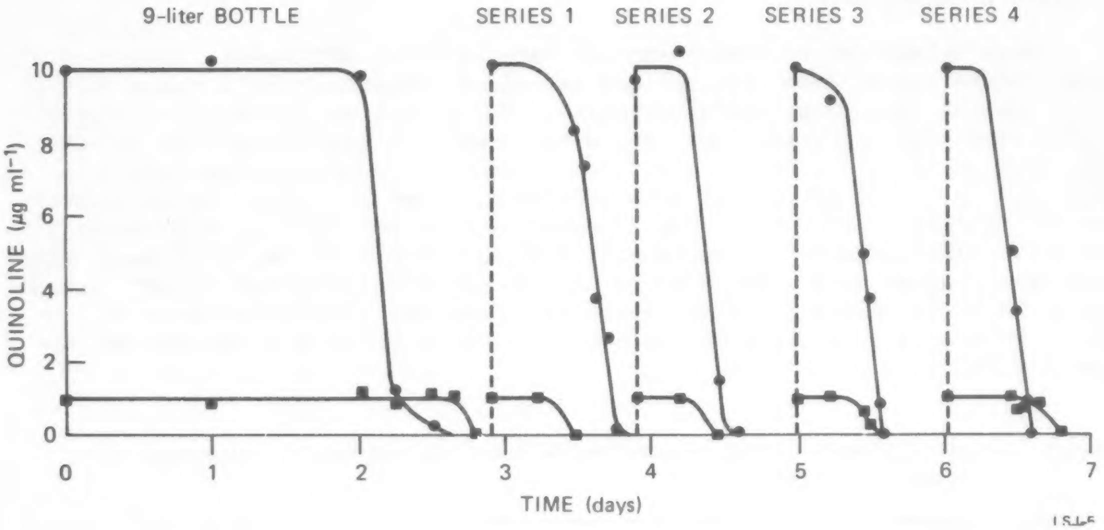


Figure 2. Cascade Batch Fermentations with a freshly developed degrading system transferred in fresh pond water media.

BENZO[f]QUINOLINE (BQ)

The results with BQ and quinoline (Q) as pollutants in Table 4 are essentially similar to those in Table 3. Q was the inducer of enzymes that degraded BQ. However, in contrast to the data obtained with DBT (Table 3), even after more than 6 weeks of induction and sub-transfer, cometabolism was still involved in rapid BQ degradation by three of the six culture mixtures that initially degraded BQ readily in the presence of Q. Again, in the presence of microbial nutrients such as glucose plus yeast extract, no degradation of BQ was observed after 6 weeks of incubation of the one water sample tested. The data in Figure 3 were obtained with one mixed culture transferred 30 times in shaker flasks containing basal inorganic salts medium plus BQ and Q. The presence of Q accelerated degradation of BQ, but with this culture mixture, only 30% of the BQ was degraded in the absence of Q. Addition of glucose plus yeast extract to the medium completely eliminated BQ degradation. One transfer in trypticase soy broth plus Q medium still resulted in BQ degradation in the absence of Q, but with several transfers, results similar to those with MP (Table 1) probably would have been obtained.

TABLE 4. DEVELOPMENT OF BENZO[f]QUINOLINE(BQ)-BIODEGRADING CULTURES IN AQUATIC SYSTEMS

Addition to water samples	Time for BQ degradation (days)	BQ degradation*
Eutrophic waters and sewage effluents		
BQ alone	15-43	2/5
BQ + quinoline (Q)	2-3	5/5
BQ + glucose + yeast extract	--†	0/1
Oligotrophic waters		
BQ alone	--†	0/1
BQ + Q	8	1/1

*Number of water samples that developed BQ-biodegrading cultures/number of different water samples tested.

†Did not develop within 6 weeks.

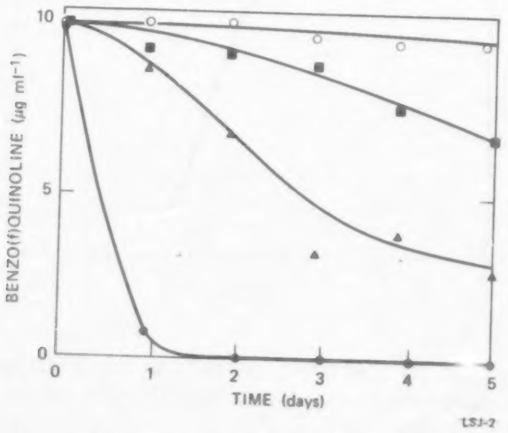


Figure 3. Biodegradation of benzo[f]quinoline inoculated with a previously isolated benzo[f]quinoline and quinoline degrading mixed culture. Supplements to inorganic salts: BQ + Q (●); BQ alone (■); BQ + glucose + yeast extract (o), and BQ + trypticase soy broth (▲).

The experiments in Figure 4 were conducted with estuarine water. Q was degraded after a lag period and BQ did not interfere with Q degradation. However, Q was necessary to induce enzymes for BQ degradation, functioning as a cometabolic substrate. Initially, BQ degradation began at about 12 d (point A) and ceased when Q was completely degraded. The addition of more Q on day 15 initiated a second degradation of BQ, commencing at about 17 d (point B). Naphthalene (N) also functioned as an inducer and cometabolic substrate in BQ degradation in estuarine water (Fig. 5).

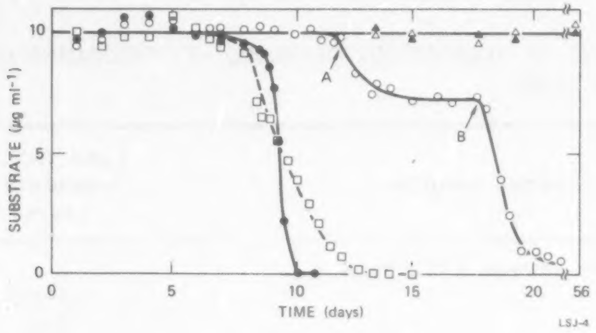


Figure 4. Biodegradation of quinoline and benzo[f]quinoline in estuarine water. Q in waters supplemented with Q alone (●) or BQ plus Q (□). BQ in waters supplemented with BQ (△) alone, BQ plus peptone (▲), or BQ plus Q (o). Additional Q was added to BQ + Q supplemented waters at 15 days.

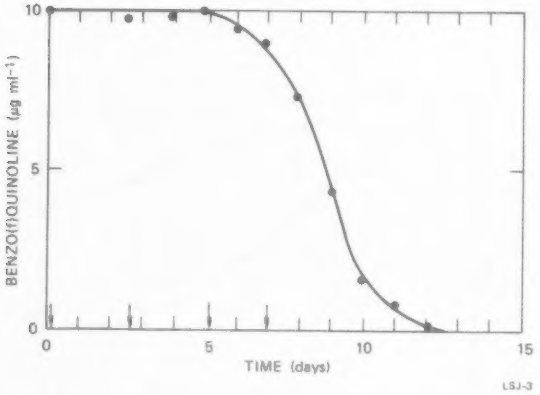


Figure 5. Biodegradation of benzo[f]quinoline in estuarine water with naphthalene as a cometabolic substrate. N (10 µg ml^{-1}) was added at 0, 2.5, 5, and 7 days (↓).

As indicated in Figure 6, development of isoquinoline(IQ)-degrading organisms in saline estuarine water appeared to be considerably slower than development of Q-degrading cultures with Q as the added substrate. IQ degradation apparently was facilitated by the presence of BQ, but this may be an artifact in that particular fermentor. BQ was not degraded after 56 days in the presence of IQ. In this instance, IQ was not a cometabolic substrate for BQ degradation.

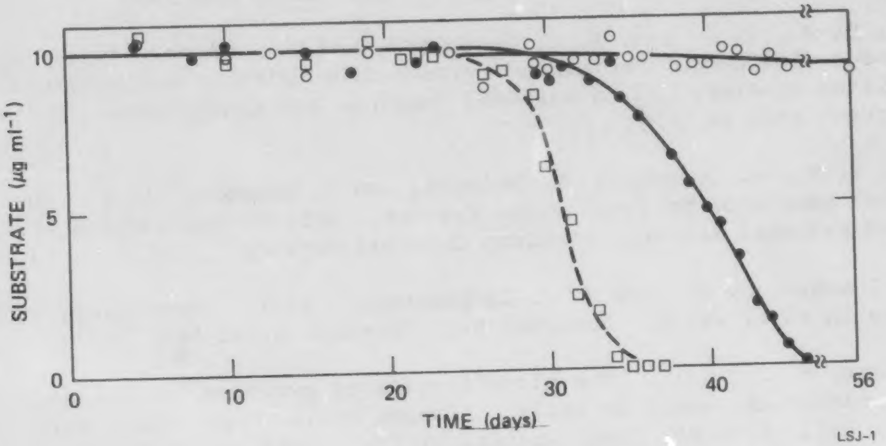


Figure 6. Biodegradation of isoquinoline and benzo[f]quinoline in est[illegible]ne water. IQ in waters supplemented with IQ alone (●) or BQ plus IQ (□). BQ in waters supplemented with BQ plus Q (○).

Data are presented here that demonstrate the problems of determining the biodegradability or recalcitrance of compounds in natural waters. These observations could be particularly significant in some ecosystems. Compounds with somewhat similar structural features can be important as inducers and cometabolic substrates in developing enrichment cultures and possibly for isolation of pure strains of organisms for studies of metabolic pathways. Naphthalene has been an excellent inducer and cometabolic substrate in degradation of the aromatic heterocyclic compounds studied in this report.

The presence of readily assimilated microbial nutrients inhibits formation and maintenance of mixed culture-degrading systems. These observations may have to be considered in assessing the effectiveness of sewage treatment plants that process domestic and industrial feeds and of ecosystems that leach organic nutrients into aqueous phases.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Bohonos, N., T.-W. Chou, and R. J. Spanggord. 1977. Some observations on biodegradation of pollutants in aquatic systems. Japan. J. Antibiot. 30(Suppl.):S275-S285.

2. Bourquin, A. W., M. A. Hood, and R. L. Garnas. 1977. An artificial microbial ecosystem for determining effects and fate of toxicants in a salt-marsh environment. Dev. Ind. Microbiol. 18:185-191.

3. Chou, T.-W., S. S. Lee, R. J. Spanggord, and N. Bohonos. 1976. Continuous-flow compartmentalized fermentation units in environmental degradation studies. 172nd National Meeting, American Chemical Society. Abstract MICR 16.

4. Chou, T.-W., R. Spanggord, E. Shingai, and N. Bohonos. 1976. The fate of methyl parathion in fresh water systems. III. Biodegradation studies. 172nd National Meeting, American Chemical Society.

5. Eichelberger, J. W., and J. J. Lichtenberg. 1971. Persistence of pesticides in river water. Environ. Sci. Technol. 5:541-544.

6. Funchess, M. J. 1917. The nitrification of pyridine, quinoline, quanidine carbonate, etc., in soils. Alabama Agric. Expt. Sta., Bull. 196; Tech. Bull. 3:65-82; Chem. Abstra. 11:3362 (1917).

7. Gibson, D. T. 1975. The microbial degradation of aromatic petroleum products, Annual Report No. 2. Prepared for Office of Naval Research under Contract No. N00014-67-A-0126-0020, University of Texas at Austin (30 June 1975).

8. Hou, C. T., and A. I. Laskin. 1975. Microbial conversion of dibenzothiophene. Dev. Ind. Microbiol. 17:351-362.

9. Hsieh, D. P. H., and M. Munnecke. 1972. Accelerated microbial degradation of concentrated parathion. Proc. IV Ferm. Symp. Ferment. Technol., pp. 551-554.

10. Knecht, A. T., Jr. 1962. Microbial oxidation of dibenzothiophene and its possible application in the desulfurization of coal and petroleum. Diss. Abst. 22:3350.

11. Kodama, K. 1977. Co-metabolism of dibenzothiophene by *Pseudomonas jianii*. Agric. Biol. Chem. 41:1305-1306.

12. Kodama, K., S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, and K. Yamada. 1970. Microbial conversion of petro-sulfur compounds. III. Isolation and identification of products from dibenzothiophane. Agric. Biol. Chem. 34:1320-1324.

13. Kodama, K., K. Umehara, K. Shimizu, S. Nakatani, Y. Minoda, and K. Yamada. 1973. Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. Agric. Biol. Chem. 37:45-50.

14. Lichtenstein, E. P., and K. R. Schulz. 1964. The effects of moisture and microorganisms on the persistence and metabolism of some organophosphorous insecticides in soils with special emphasis on parathion. J. Econ. Entomol. 57:618-627.

15. Maleszewska, J. 1974. Degradation of methyl parathion by microorganisms occurring in surface water and sewage. Pol. Arch. Hydrobiol. 21:163-171.

16. Munnecke, D. M. 1976. Enzymatic hydrolysis of organophosphate insecticides, a possible pesticide disposal method. Appl. Environ. Microbiol. 32:7-13.

17. Munnecke, D. M., and D. P. H. Hsieh. 1974. Microbial decontamination of parathion and *p*-nitrophenol in aqueous media. Appl. Microbiol. 28:212-217.

18. Munnecke, D. M., and D. P. H. Hsieh. 1975. Microbial metabolism of a parathion-xylene pesticide formulation. Appl. Microbiol. 30:575-580.

19. Munnecke, D. M., and D. P. H. Hsieh. 1976. Pathways of microbial metabolism of parathion. Appl. Environ. Microbiol. 31:63-69.

20. Nakatani, S., T. Akasaki, K. Kodama, Y. Minoda, and K. Yamada. 1968. Microbial conversion of petro-sulfur compounds. II. Culture conditions of dibenzothiophene-utilizing bacteria. Agric. Biol. Chem. 32:1205-1211.

21. Naumann, K. 1967. Decomposition of parathion by soil bacteria. Phytopathol. Z. 60:343-357.

22. Paris, D. F., D. L. Lewis, and N. L. Wolfe. 1975. Rates of degradation of malathion by bacteria isolated from aquatic systems. Environ. Sci. Technol. 9:135-138.

23. Robbins, W. J. 1916. The cause of the disappearance of coumarin, vanillin, pyridine and quinoline in the soil. Science 44:894-895.

24. Sagardia, F., J. J. Rigau, A. Martinez-Lahoz, F. Fuentes, C. Lopez, and W. Flores. 1975. Degradation of benzothiophene and related compounds by a soil pseudomonas in an oil aqueous environment. Appl. Microbiol. 29:722-725.

25. Sethunathan, N., and T. Yoshida. 1973. Parathion degradation in submerged rice soils in the Philippines. J. Agric. Food Chem. 21:504-506.

26. Sidderamappa, R., K. P. Rajaram, and N. Sethunathan. 1973. Degradation of parathion by bacteria isolated from flooded soil. Appl. Microbiol. 26:846-849.

27. Smith, J. H., W. R. Mabey, N. Bohonos, B. R. Holt, S. S. Lee, T.-W. Chou, D. C. Bomberger, and T. Mill. 1977. Environmental pathways of selected chemicals in freshwater systems. Part I: Background and experimental procedures. EPA Publ. No. EPA-600/7-77-113. Part II: Laboratory studies. EPA Publ. No. EPA-600/7-78-074.

28. Walker, J. D., and R. R. Colwell. 1974. Microbial petroleum degradation: use of mixed hydrocarbon substrates. Appl. Microbiol. 27:1053-1060.

29. Walker, J. D., R. R. Colwell, and L. Petrakis. 1975. Microbial petroleum degradation: application of computerized mass spectrometry. Can. J. Microbiol. 21:1760-1767.

30. Yamada, K., Y. Minoda, K. Kodama, S. Nakatani, and T. Akasaki. 1968. Microbial conversion of petroleum compounds. I. Isolation and identification of dibenzothiophene utilizing bacteria. Agric. Biol. Chem. 32:840-845.

31. Yasuno, M., S. Hirakoso, M. Sasa, and M. Uchida. 1965. Inactivation of some organophosphorous insecticides by bacteria in polluted water. Japan. J. Exp. Med. 35:545-563.

METABOLISM OF CYCLOPROPANE CARBOXYLIC ACID BY BACTERIA

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ABSTRACT

A *Corynebacterium* strain was isolated from soil by enrichment at the expense of cyclopropanecarboxylate (CPC). Substrate utilization and oxygen uptake patterns indicate that the pathway for use of CPC by this bacterium is inducible and differs from that previously described for the fungus, *Fusarium*, and liver. Properties of mutants that have lost the ability to grow at the expense of CPC and their revertants implicate in the oxidation of CPC portions of a pathway previously described as functional in the degradation of valine by bacteria. Measurement of enzyme activity in crude extracts confirms this conclusion. The mode of initial attack upon the cyclopropane ring remains obscure.

Alicyclic (cycloparaffin and naphthene) compound occur widely in organisms and in organic deposits, such as petroleum, derived from them. Some of the most interesting of such compounds are cyclopropane and its derivatives. The three-membered ring structure present in these compounds is highly strained, and displays certain chemical similarities to a simple carbon-carbon double bond; for instance, it is readily reactive with bromine. Further, the unsaturated analog of cyclopropane, namely cyclopropene, has been shown to serve as substrate for bacterial nitrogenase, an enzyme capable of reducing *triple* bonds (6).

Cyclopropane carboxylic acid (CPC) is of interest as a model compound and as a substance, the metabolism of which is of interest in its own right. Compounds that are derivatives of, contain, or may yield CPC occur in petroleum, pyrethrins, hypoglycins, bacterial fatty acids, a miticide now being developed for commercial use (cycloprate: the hexadecyl ester of CPC), and other compounds proposed for use or in use. Examples are a coccidiostat (cyproquinate), an herbicide (cypromid), a tranquilizer (prazepam), and several narcotic antagonists (naltrexone, cyclazocine, and oxilorphan) (11, 13).

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CPC can serve as sole carbon and energy source for growth of some microorganisms with complete degradation of the cyclopropane ring. Two mechanisms for this degradation have been identified. Tipton and co-workers (5, 14) found that *Ochromonas danica* and *Tetrahymena pyriformis* mineralize cyclopropane derivatives by a simple modification of β-oxidation, in which the ring is treated as a double bond. Quite a different mechanism was identified in the fungus *Fusarium oxysporum*. Chung and co-workers (3, 9, 10) showed that cleavage of the ring occurred by direct addition of the elements of water across one of the bonds adjacent to the α-carbon. This attack yields γ-hydroxybutyric acid, which is then degraded by known routes. The present paper reports still another route for degradation of the cyclopropane ring in bacteria.

MATERIALS AND METHODS

ORGANISM AND CULTURE METHODS

A *Corynebacterium* sp., designated strain CPC-1, was isolated by liquid enrichment using a mineral medium (7) with CPC as sole source of carbon and energy. For other purposes this medium was modified by inclusion of 1% (w/v) Ionagar No. 2 (Colab, Inc.) and by replacement of 0.2% Na-CPC by other carbon sources. Growth was measured at 30°C in side arm-fitted Erlenmeyer flasks using a Klett colorimeter (No. 66 filter).

MUTAGENESIS AND MUTANT ISOLATION

Mutants were isolated from independent cultures following treatment with various concentrations of 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich Chemical Co.) in growth medium, several generations of growth in mutagen-free medium for expression, and plating on solid medium containing 0.2% Na-CPC and 0.01% Na-succinate. Small colonies were picked and screened for loss of the ability to grow at the expense of CPC.

CHEMICALS

All organic chemicals used as substrates were obtained from Sigma Chemical Co., with the exception of *iso*-butyric acid (Fisher Scientific Co.), CPC, 3-hydroxybutyric acid, methacrylic acid (Aldrich Chemical Co.), 1-hydrocybutyric acid (K & K Laboratories), cyclopentane carboxylic acid (PCR Incorporated) and cyclobutane carboxylic acid (ICN Pharmaceuticals, Inc.). Inorganic chemicals were all of reagent grade. *Iso*-butyryl-CoA was synthesized by the mixed anhydride method (1) using freshly distilled reagents.

ANALYTICAL METHODS

Gas chromatographic measurement of CPC and other volatile acids in acidified (H_3PO_4) aqueous media was performed with a Hewlett-Packard Model 402 instrument fitted with a 2 m x 2 mm "U" tube column of 10% SP-1200, 1% H_3PO_4 on 80-100 mesh Chromosorb WAW (Supelco, Inc.) at 105°C.

Oxygen uptake was determined polarographically with resting cell suspensions at 30°C in the buffer portion of the mineral growth medium (above) using a Yellow Springs Instrument Co. Model 53 oxygen monitor connected to a Hewlett-Packard Model 7128A recorder.

Cell extracts for enzyme assay were prepared by sonic extraction at 0°C of a 10% wet weight suspension of cells in 0.1 M phosphate buffer (pH 7.2). Following extraction, unbroken cells and debris were removed by centrifugation at 10,000 g for 10 min at 0°C. *Iso*-butyryl-CoA dehydrogenase was measured according to Green et al. (2). Protein in extracts was measured using Pardee's modification of the biuret reagent (4); bovine serum albumin (Sigma Chemical Co.) served as standard.

RESULTS AND DISCUSSION

CPC is metabolized by strain CPC-1 with the accumulation in the culture fluid of compounds detectable by gas chromatography using a flame ionization detector. Accordingly, it was decided to search for evidence of the pathway being used by means of a study of blocked mutants, substrate utilization, and oxygen uptake patterns. Before it was possible to undertake either of the latter two approaches, it was necessary to consider which compounds might be intermediates in the mineralization of the cyclopropane ring.

Cleavage of the cyclopropane ring of CPC could yield various products, depending on the site and mode of attack. These are summarized in Figure 1. Hydrogenation of either of the bonds adjacent to the α-carbon would give *n*-butyrate, while hydrolysis of those bonds would give either α-hydroxybutyrate or γ-hydroxybutyrate (Chung [10] found that *F. oxysporum* produces γ-hydroxybutyrate). On the other hand, attack upon the bond opposite the α-carbon yields *iso*-butyrate or a related compound such as methacrylate or 3-hydroxyisobutyrate. The formation of metacrylate from CPC would be an internal arrangement, in which the strained ring configuration is resolved into a true double bond. Such a dismutation could also involve one of the adjacent bonds, yielding 2-butenoate or 3-butenoate. Still another possibility is the oxygenation of the cyclopropane ring to the corresponding lactone (butyrolactone).

The results of growth experiments presented in Table 1 indicate that degradation of CPC proceeds neither by attack at a bond adjacent to the α-carbon, or by lactonization of the ring. Neither *n*-butyrate or its hydroxy derivatives supported growth of the bacterium. Butyrolactone was not attacked. In contrast, *iso*-butyrate, metacrylate, valine and 2-oxoisovalerate all supported growth of the organism at rates equal to or slightly greater than the growth rate on CPC. The unsaturated acid, 2-butenoate, also supported growth, though only after a lengthy lag and at sluggish rates. Growth on 3-butenoate was attended by no lag, but again growth was extremely slow.

To distinguish between intermediates involved in degradation of CPC and fortuitous use of putative intermediate compounds, measurement of oxygen uptake in suspensions of resting cells grown on these compounds was examined.

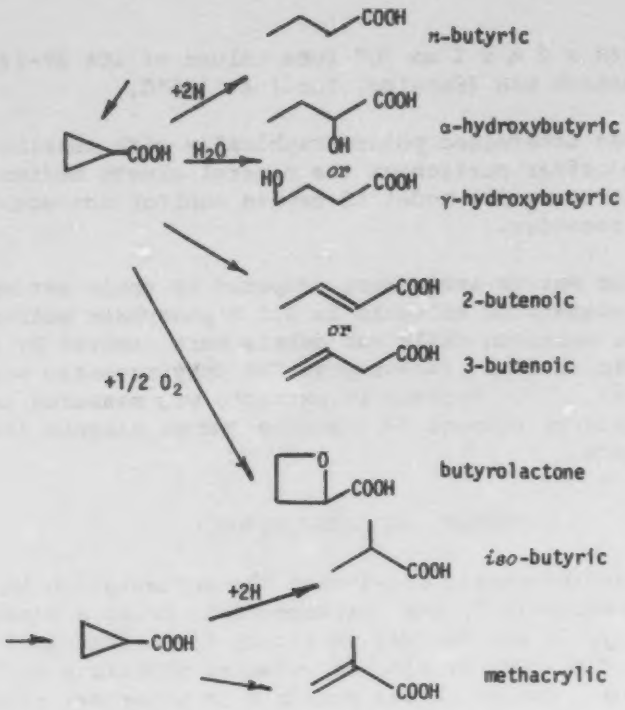

Figure 1. Possible modes of attack upon cyclopropanecarboxylic acid (CPCO, and the resulting products. The arrows indicate the bond attacked. The unlabeled arrows indicate ring cleavage via rearrangement. *Fusarium oxysporum* engages in a hydrolytic fission of the cyclopropane ring with γ-hydroxybutyric acid as product (10).

TABLE 1. GROWTH OF *Corynebacterium* CPC-1 AT THE EXPENSE OF POSSIBLE INTERMEDIATES IN CPC METABOLISM[a]

Cyclopropane carboxylate	++	2-oxoisovalerate	++
Glutarate	+	Valine[b]	++
Succinate	++	α-hydroxybutyrate	-
Iso-butyrate	++	γ-hydroxybutyrate	-
Methacrylate	++	2-butenoate	+
n-butyrate	-	3-butenoate	+
		Butyrolactone	-

[a] Growth was measured turbidimetrically in liquid mineral medium as indicated in Materials and Methods. Substrates of organic acids were supplied at a concentration of 0.2% (w/v) as the sodium salts.

[b] Supplied as DL-valine.

The results are summarized in Table 2. Several points deserve special attention. First, utilization of CPC, *iso*-butyrate or methacrylate is strictly inducible. When each of these substrates is offered to starved cells grown on succinate, O_2 consumption is not stimulated, but remains at background level. However, when each of these substrates is offered to starved cells previously grown on CPC or on *iso*-butyrate, O_2 consumption is immediately stimulated. In contrast, when cells are grown on either 2-butenoate or 3-butenoate, supply of CPC, *iso*-butyrate or methacrylate to starved cells produces no stimulation of O_2 consumption, i.e., neither induces oxidation of CPC, *iso*-butyrate or methacrylate. Likewise, growth of cells on CPC or on *iso*-butyrate show a slight stimulation of O_2 consumption when given 3-butenoate, but never reach O_2 consumption rates comparable to those elicited by succinate.

TABLE 2. RELATIVE RATE OF O_2 CONSUMPTION BY CPC-1 CELLS[a]

Test substrate[b]	Cells grown at the expense of:				
	Succinate	2-butenoate	3-butenoate	CPC	*iso*-butyrate
Succinate	84	10	41	80	83
2-Butenoate	30	35	--[c]	9	10
3-Butenoate	40	--[c]	60	25	15
CPC	10	9	7	76	78
Iso-butyrate	10	10	10	81	82
Methacrylate	10	8	9	78	79

[a] Microliters of O_2 taken up by a turbidimetrically standardized suspension per unit time at 30°C. Cells were harvested by centrifugation at room temperature and starved by incubation in growth medium to reduce the endogenous level of O_2 uptake for a standard time. Measurements were performed as indicated in Materials and Methods.

[b] Supplied as the sodium salts at a final concentration of 0.2% (w/v).

[c] Not done.

Since patterns of induction appear to eliminate 2-butenoate and 3-butenoate as intermediates in CPC degradation, attention is focused on *iso*-butyrate and methacrylate. Sokatch (12) and others have shown that in several bacterial strains, degradation of valine proceeds via the pathway shown in Figure 2, in which 2-oxoisovalerate, *iso*-butyrate and methacrylate are intermediates.

A mutant of strain CPC-1 was isolated, following mutagenesis, that had lost the ability to grow at the expense of CPC. It was found to have simultaneously lost the ability to grow with *iso*-butyrate, methacrylate and valine, although growth on succinate was unimpaired. When large numbers of succinate-grown mutant cells are plated onto agar with either CPC or valine, a small number of presumed revertant colonies appear following incubation. Forty-seven revertant colonies were picked from CPC and transferred to fresh valine plates; 100 revertant colonies were picked from the valine plates and transferred to fresh CPC medium. When growth had occurred, the plates were examined: 100% of the CPC revertants had regained the ability to grow on valine, and 100% of the revertants selected for regain of ability to grow on valine had simultaneously regained the ability to grow with CPC.

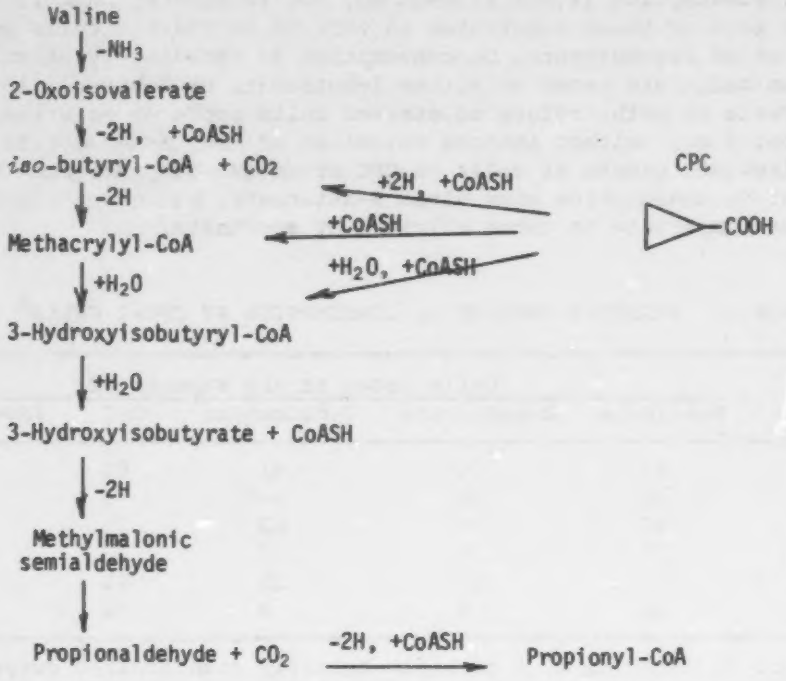

Figure 2. The convergence of valine and CPC metabolism proposed to occur in *Corynebacterium* strain CPC-1 (modified from Sokatch [12]). It is not known whether activation by coenzyme A precedes ring fission, or which of the three possible ring fission mechanisms occur.

Direct enzymatic evidence for participation of *iso*-butyrate as an intermediate in CPC degradation was sought by assay of *iso*-butyryl-CoA dehydrogenase in extracts of succinate-, CPC-, and *iso*-butyrate-grown cells. The results are given in Table 3. It is clear that *iso*-butyryl-CoA dehydrogenase is elevated in CPC-grown cells as in *iso*-butyrate-grown cells.

TABLE 3. ACTIVITY OF *iso*-BUTYRYL-CoA DEHYDROGENASE IN CRUDE EXTRACTS OF *Corynebacterium* CPC-1[a] (nmoles/hr/mg protein)

Cells grown on CPC	11.5
Cells grown on *iso*-butyrate	13
Cells grown on succinate	2

[a] Cells were harvested during exponential growth, extracted, and enzyme activity and protein measured as indicated in Materials and Methods.

In light of the results described above, we conclude that *Corynebacterium* CPC-1 degrades CPC by attack on the cyclopropane ring opposite the α-carbon. Subsequent metabolism occurs via a scheme previously described for valine degradation by bacteria (12; Fig. 2).

The bacterial metabolism of higher alicyclic hydrocarbons and their derivatives generally entails initial conversion of the compound to the corresponding alicyclic ketone and subsequent introduction of an oxygen atom adjacent to the oxidized carbon in a mixed-function oxidase-mediated reaction. The lactones formed in this way hydrolyze spotaneously or in an enzyme-catalyzed process to yield aliphatic hydroxy acids susceptible to further metabolism by conventional routes (8). CPC is an apparent exception to this pattern either because of the presence of the carboxyl function, which may prevent the occurrence of some step of the usual process, or the size and/or configuration of CPC may cause it to be handled initially as an aliphatic compound. The occurrence of CPC activation either as the carnitine (*Fusarium* [3]) or CoA (*Corynebacterium* CPC-1) thioester, if it precedes ring cleavage, suggests that this latter explanation may be true. The higher cycloalkanes are metabolized by bacteria without initial activation as CoA thioesters.

However, the proposed pathway does have certain general features common to other pathways for the degradation of alicyclic hydrocarbons. First, the pathway is inducible. Second, there is a high degree of specificity as to substrates. *Corynebacterium* CPC-1 is totally unable to grow on any other alicyclic compound tested in the C_4 to C_7 range. Third, the portion of the proposed degradative pathway unique to CPC metabolism is very short, in this case only two steps (activation and ring cleavage) long. It is generally true, as here, that degradation of alicyclic compounds proceeds by conversion of the alicyclic compound to a form that can feed into some commonplace central metabolic pathway. All of these points emphasize the key role of the initial steps involving primary attack upon the substrate molecule, as well as the regulatory mechanisms that govern their synthesis and the synthesis of enzymes functional in later steps.

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LITERATURE CITED

1. Goldman, P., and P. R. Vagelos. 1961. The specificity of triglyceride synthesis from diglycerides in chicken adipose tissue. J. Biol. Chem. 236:2620-2623.

2. Green, D. E., S. Mii, and H. R. Mahler. 1954. Studies on the fatty acid oxidizing system of animal tissues. III. Butyryl coenzyme A dehydrogenase. J. Biol. Chem. 206:1-12.

3. Guilbert, C. C., and A. E. Chung. 1974. Metabolism of cyclopropane carboxylic acid. A new role for carnitine. J. Biol. Chem. 249:1026-1030.

4. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. III. Biuret method, p. 450-451. *In* S. P. Colwick and N. O. Kaplan (eds.), Methods in enzymology, Vol. 3. Academic Press, New York.

5. Magat, W. J., and C. L. Tipton. 1970. Oxidation of cyclopropane fatty acids by *Ochromonas danica*. Arch. Biochem. Biophys. 141:353-355.

6. McKenna, C. E., M.-C. McKenna, and M. T. Higa. 1976. Organic probes of nitrogenase. J. Amer. Chem. Soc. 98:4657-4659.

7. Palleroni, N., and M. Doudoroff. 1956. Mannose isomerase of *Pseudomonas saccharophila*. J. Biol. Chem. 218:535-548.

8. Perry, J. J. 1977. Microbial metabolism of cyclic hydrocarbons and related compounds. CRC Crit. Rev. Microbiol. 5:387-412.

9. Schiller, J. G., and A. E. Chung. 1970. The metabolism of cyclopropane carboxylic acid. J. Biol. Chem. 245:5857-5864.

10. Schiller, J. G., and A. E. Chung. 1970. Mechanism of cyclopropane ring cleavage in cyclopropane carboxylic acid. J. Biol. Chem. 245:6553-6557.

11. Schooley, D. A., G. B. Quistad, and L. E. Staiger. 1978. Cyclopropane carboxylic acid: chain elongation to ω-cyclopropyl fatty acids by mammals and plants. Science 199:544-545.

12. Sokatch, J. R. 1968. Bacterial physiology and metabolism, p. 180-182. Academic Press, New York.

13. Tanaka, K. E., A. Kean, and B. Johnson. 1976. Jamaican vomiting sickness. New England J. Med. 295:461-467.

14. Tipton, C. J., and N. M. Al-Shatir. 1974. The metabolism of cyclopropane fatty acids by *Tetrahymena pyriformis*. J. Biol. Chem. 249:886-889.

DISCUSSION

BIOCHEMISTRY OF MICROBIAL DEGRADATION

TIEDJE: Dr. Chapman, do you see any reason why marine organisms or a marine environment would effect a different type of metabolism?

CHAPMAN: Recent studies have drawn attention to the fact that hydrocarbons are degraded with increasing difficulty at increasing levels of salinity. Furthermore, studies by Dr. Bourquin on the degradation of the compound nitrilotriacetic acid (NTA) by a freshwater organism have demonstrated that there is a serious impairment to the degradation of NTA when freshwater organisms are present in estuarine environments. Based on those two examples, I would merely say that we know very little about the constraint of salinity upon the degradation of chemicals by freshwater and soil microorganisms, and I feel that there is a great deal to be learned. Possibly even more complex alternatives are going to turn up from a study of organisms which have the marine environment as their specific habitat and are not responsible to growth under freshwater conditions.

CHAPMAN: Dr. Bollag, fungi are notorious for making secondary metabolites and they are known formers of coupled products when they are the symbionts in lichens. A number of the products that are formed from carbaryl are evidently hydroxylation products. Furthermore, they are coupled products, oxidative coupling products. Do you think there is a relationship that characterizes these fungi or relates these conversions to their normal process of secondary metabolism?

BOLLAG: Well, they are not mainly hydrolytic compounds, they are phenolic-type compounds. My opinion is that the nature of these phenolic compounds represents a special group of metabolic products. We assume that they are active in secondary metabolic activity and that the fungi have a major function in this kind of polymerization process.

GIBSON: You said the enzyme was an extracellular enzyme, Dr. Bollag. It seems to me those products are very similar to what you get in phenol couplings with enzymes like horseradish peroxidase and laccases; I think you mentioned yours was a laccase. Do you have any evidence to support this?

BOLLAG: That's right, we did find it to be a laccase. That means we purified and identified it as a laccase, but it is also possible that a peroxidase can catalyze this reaction.

CHAPMAN: Dr. Alexander, do you think that the non-growth substrates can also serve as the inducers of the enzymes which may or may not be responsible for their degradation? I think much of the biochemical and microbiological basis for cometabolism resides, as you've quite correctly stated, between the structural relationships of growth substrates and non-growth substrates. You mentioned, quite correctly I believe, the importance of evaluating enzyme specificity in this regard, but there's a second very important barrier that has to be overcome and that is the de-repression of appropriate enzymes by such molecules. Would you care to discuss this?

ALEXANDER: The problem of the induction or de-repression is a very real one in the understanding of the biochemistry/microbiology of cometabolism. I agree that the work ought to be done, and we have little information on this matter. We have tried to estimate whether induction was significant by adding biodegradable analogs to a natural environment. This work was done in freshwater, sewage, and soils, using a number of different compounds--benzoates, diphenylmethanes, and others. In no case did we find much of a change. Probably the cometabolizing organisms are not benefitted greatly by the substrates we think they ought to respond to. The organisms that respond to such additions are probably pseudomonads, flavobacteria or other organisms which quickly utilize the added compound, which is then all gone by the time the cometabolizing populations begin to metabolize the chlorinated molecules to a significant extent.

CHAPMAN: Undoubtedly, it is particularly important to determine what sort of things are going on in these processes, and if one can achieve conditions where one can direct or facilitate degradation by a suitable choice of a growth substrate, then I think it's important that we understand these relationships so that we can direct or facilitate degradation in this fashion.

ALEXANDER: It is also important to know what substrates do to natural populations, as well as obtaining the biochemical information. These population studies will not be easy to do well.

TIEDJE: Another consideration here should be the phenomenon of catabolite repression, because I think a lot of substrates that we might add in nature could often have a negative effect on degradation of materials.

ALEXANDER: In dealing with a microbial community of many populations, we tend to extrapolate too readily from pure cultures to natural environments. The organism which is affected by catabolite repression may not be the organism involved in cometabolism. Again, let me stress that it is important to get the information on the biochemistry and microbiology, and similarly on the ecology of the responsible species.

TIEDJE: You made a statement that was a little surprising to me. You said that the evidence for cometabolism in nature is shaky, and I want to know what kind of evidence you're looking for that would be more solid. What kind of phenomena?

ALEXANDER: Something other than our inability to isolate the responsible organisms. It would be satisfying to show that the disappearance of the molecules was parallel to no increase in population of the active organisms. Alternatively, if C^{14} organic compounds subject to cometabolism are not converted to C^{14} cellular products in the environment, then likely cometabolism is occurring.

RAYMOND: Dr. Alexander, I can't quite fathom the comment you made that cometabolism will not lead to CO_2. Did I misinterpret that?

ALEXANDER: I should retract somewhat. Cometabolism will not lead to a major conversion of the substrate, but a cometabolic decarboxylation reaction would lead to CO_2. But cometabolism will not give, as with glucose in a natural environment, 6 moles of CO_2 for one mole of glucose, or 5, considering carbon-assimilatory processes.

ZAJIC: My questions really have to do with the carbon monoxide data. One, I wonder whether the reaction was run under aerobic or anaerobic conditions, and two, whether any gaseous hydrogen was produced. Also, I don't know what happened to Carboxymonas in the recent edition of Bergey's Manual; maybe they don't exist any more, but the older data by A. J. Kluyver in 1945-46 indicated that oxidation of carbon monoxide occurred under anaerobic conditions. There was a reductive cleavage of water, and some hydrogen was produced. I wondered if this might not be what you're seeing.

ALEXANDER: Carbon monoxide was oxidized under anaerobic and aerobic conditions. The generic name you cited does not appear in the current volume. We have not determined whether hydrogen is evolved.

ZAJIC: Many times with carbon monoxide oxidation to CO_2 and with hydrogen release you see methane as the end product. That's just a comment; I want to ask something else. I wonder if we have indirect evidence of cometabolism. Natural products like lignin are very difficult to oxidize with pure cultures in the laboratory. Yet in nature, we find turnover is slow but of a far greater rate than predictions from anything we have been able to accomplish in the laboratory to date. In making another example, the alphaltene materials from crude oil, if put in the soil, are oxidized to only a limited extent. It is almost impossible to find a single culture to metabolize it, yet a long-term study in the soil shows something happening that we've never been able to do in the laboratory using more exacting controlled conditions. These are somewhat indirect examples, but things are happening in the environment that we haven't been able to approach or handle in the laboratory under controlled conditions. What is your reaction to an indirect type of evidence?

ALEXANDER: Such arguments are quite tenuous, although the facts may be correct. In the case of depolymerization of lignin in pure culture, it's quite possible that the monomeric fragments build up, and these may be quite toxic; but in a mixed culture in a natural ecosystem, there would be populations which would destroy these phenolic or methoxy compounds. Also, the initial populations depolymerizing lignin could be growth-factor requiring, but in nature, organisms produce the growth factors for the depolymerizing species.

MATSUMURA: I am interested in your definition of cometabolism. I understand that certainly in related chemicals we can talk about cometabolism. How about metabolism by quite general enzymes, such as hydrolytic enzymes, excreted outside of the cell, hydrolysis on something like carbaryl by exo-enzymes--do you call this degradation or cometabolism?

ALEXANDER: You're trying to nail me to the wall on semantics, but I have a very low semantic sensitivity. For the sake of the definition, I would say the organism is not cometabolizing the compound. On the other hand, a product is accumulating. Such accumulations occur with compounds which contain quaternary carbons. The organism keeps attacking the molecule until it gets close to the quaternary carbon and then it stops. The consequence is the important point, whatever the phenomenon is called.

GIBSON: In the batch system, Dr. Chou, did you look for accumulation of products, or did you just measure substrate disappearance?

CHOU: In most of these experiments, I measured disappearance of substrate; however, in the mixed culture, in our experiments it seems to me the products are degraded further, maybe as a function of the mixed organisms.

GIBSON: You may get extensive degradation?

CHOU: Yes.

RAYMOND: Dr. Gibson, I can't pass up the opportunity to explore further into this business of products of cometabolism accumulating in nature, which Dr. Alexander has alluded to. Our evidence, certainly with hydrocarbons, is that products do not accumulate in nature from cometabolism, or rather from the cooxidation. Admittedly, we have not isolated all the possible cultures from that particular aquatic environment. Because we, like you, have had a lot of negative studies using a pure compound and attempting to find a microorganism in the environment which utilizes it for growth and energy. Most of the time we don't find them. What we do find, in nature, is, if we first add a substrate that can be used for growth and energy, then introduce the second (nonutilizable) substrate, the second is oxidized. Furthermore, in freshwater and soil environments, we don't see end products accumulating. I don't know that this is proof, but certainly we have looked exhaustively for end products in these systems. Perhaps some of them are tied up or conjugated so that we don't see them, but with our extractive methods we don't see them. I think this is part of what we have to establish this week: whether the methods we use to determine biodegradation are satisfactory.

ALEXANDER: I think there are two possible explanations. First, if an organism can't be isolated it does not mean that the reaction is necessarily cometabolic. Second, as in the case of *p*-chlorophenylacetic acid in a two-culture system, possibly the initial reaction is cometabolic and the product of the initial reaction will support growth; therefore, there is no accumulation.

RAYMOND: Are you still insisting, though, that if you cannot find microorganisms, at least by identifiable names, to utilize certain materials, or if we can't find in the laboratory that these materials are cometabolized, that therefore they are not biodegradable. Therefore, they are dangerous materials because they will accumulate in nature. That cannot be the gist of this removal from nature, the way I understand it.

ALEXANDER: I focus on the environment first and on the microbiology second. If there is no accumulation in nature, one would not explain the disappearance by cometabolism. It may be that cometabolism is occurring but the product would not be found. I suggest cometabolism may occur if the reaction is slow and there is an accumulation. That does not mean that accumulation is the only consequence of cometabolism in nature. My microbiology begins with the observation in nature and then we work with cultures whereas many of my colleagues prefer to begin with cultures and hope their data are relevant in nature.

GIBSON: I think if we understand what is going on, it doesn't really matter what term we ascribe to the phenomenon.

ALEXANDER: I agree, Dave, we have enough problems without semantics. There is nothing particularly unique in the question of cometabolism. It is inappropriate to worry about terminology in important environmental issues. If we understand what is going on and we can use biochemical information to predict what will happen in nature, that should be the focus of a microbiologist.

GIBSON: You suggested that there were possible hazards of carcinogens produced in the environment, for example, nitrosamines. Aren't nitrosamines reactive alkylating agents? Are they going to hang around long enough to be dangerous?

ALEXANDER: We have put dimethyl, dipropyl, diethyl nitrosamines into soils, water and sewage. In fresh water, there was no disappearance at all over four months. In the other environments about half of the concentration disappeared and the rest stayed in the system. So far as we know, they are quite persistent. Debra Raymonds found that nitrosamines can be taken up by plants (no one eats the soil as a rule). They can also move through soil into ground water. So, at least with the dialkyl nature of the amines and the N-alkyl diethanolamine, I'd be very concerned. They do seem to be reasonably persistent molecules. There are animal metabolism studies which suggest you can get a dealkylation of molecules enzymatically, but not at natural population levels of microorganisms.

GIBSON: They must be metabolized into active carcinogens by animals that ingest them. Is that correct?

ALEXANDER: I would assume so. They can then become alkylating agents after consumption.

GIBSON: Have these nitrosamines that you mentioned been tested in the Ames test?

ALEXANDER: Well, I am sure they have been tested in Ames test. They have been tested in animals and they are carcinogens.

IVANOVICI: In cometabolism studies, it seems useful to assess the growth state of the microorganisms you are working on as to whether or not they are growing. A lot of microorganisms won't grow even though they remain viable. So you don't really know what's happening to the energy. Have you ever considered measuring ATP as an indicator of microbial growth state, by an adenylate charge ratio? And if you had, what sorts of results did you obtain? If you haven't measured it, might you consider measuring it, because it could give you lots of very useful information about the growth state of the organism?

ALEXANDER: Well, we have considered it, but we haven't done the experiment. It would be interesting to see if there is any such benefit to the organism. We have, in looking at heterotrophic oxidation of nitrogen compounds, calculated what the energy (end kilocalories) would be if the organism was getting all the energy by converting ammonium to nitrate. It would be trivial by comparison with the cell yield of the organism. But we haven't done the experiment.

GIBSON: You said that the list of chemicals under TOSCA was almost bizarre, but you never mentioned any of them. What kind of things are we faced with here?

ALEXANDER: I think that I shall leave that matter to Dr. Stern. However, the list is enormous, and if one considers the number of precursors used for the major biosynthetic reactions and the solvents involved, one obtains some idea of the possibilities. The focus of attention should not be with compounds that are of interest biochemically, but those on large quantities produced by industries. The ten most wanted list is public information. That would include cresols, xylenes, nitrobenzenes, octachlorobutadiene, and pthalates.

STERN: The list was compiled on at least three criteria: 1) production volume, 2) distribution of material, and 3) structural relationships to materials which have been shown to have some hazardous effects. Although the list is long, it doesn't mean everything is subject to regulation. It means merely that they are going to be given a closer scrutiny.

GIBSON: In terms of structure, then there is nothing unusual. I got the impression we are going to be looking at some bizarre chemicals and I was getting nervous about our lack of information.

ALEXANDER: No, if you consider the precursors for major polymers, and the major solvents, it is evident that TOSCA is concerned with chemicals we usually do not study biochemically.

GIBSON: You did follow it up by saying we must look into new areas of biochemical processes. I would be interested to know what they are.

ALEXANDER: At the moment we should consider the general approaches for scientists to follow to get reasonable information both from purely laboratory studies with pure cultures and from model ecosystem studies.

COONEY: I have a perhaps naive view of many aquatic microorganisms living forever on the edge of starvation in a dilute nutrient environment. I wonder if you would comment on induction in this environment. It is difficult to envision induction as a widespread phenomenon, since it would require the synthesis of new enzymes.

ALEXANDER: This topic is outside my field of competence, although we have been concerned with problems of very low concentrations of synthetic chemicals. Problems of induction and of whether diffusion of substrates to the cell is sufficiently rapid to provide for maintenance energy and for the cell to multiply are real. It is possible the chemical concentration is so low in natural environments that there is no appreciable biodegradation. There could be bioaccumulation and consequent protection from biodegradation, because the chemical is biomagnified in the tissues; it may never be exposed to microorganisms outside.

PRITCHARD: I would like to make two comments relative to what has just been said. One, there is a report by Horvichi et al. (1962) which showed that by growing *E. coli* in a chemostat at very low nutrient concentrations and at very low dilution rates over long periods of incubation with lactose as the sole source of carbon and energy, a mutant strain of *E. coli* could be selected that was constitutive for lactose metabolism. At these low dilution rates and low substrate concentrations the constitutive mutant outcompeted the inducible parent. I think this points to the possibility that the physiology of the so-called "brink of starvation type bacteria" may be unique, and the more we can find out about their physiology, the more capable we will be at asking questions about the role of these bacterial types in natural transformation processes. The second point I'd like to make is directed toward Peter Chapman. Considering the staggering number of compounds that the Office of Toxic Substances is handling, are we in a position to begin making significant generalizations about the ability of microorganisms to oxidatize or metabolize these compounds? If we can categorize these compounds into potentially biodegradable or non-biodegradable, should we begin to emphasize the ecosystem or environmental effects on degradation processes rather than on the mechanistic aspects?

CHAPMAN: Hap, I think there is a significant kicker in your question--you asked whether one can make significant predictions? I have no doubt, from the vast accumulated information we have on biodegradation, that we can identify functional groups in natural and synthetic molecules which we can predict what reactions they will undergo. The next step is to say whether those reactions do, in fact, take place in certain environments. Clearly, if such a reaction is seen, say, to be catalyzed by a mono- or a dioxygenase, it will happen in aerobic environments, but not in anaerobic environments. So much of this prediction has to be conditioned by relationships within prevailing environments, habitats and their constituent microflora. One can say, yes, these reactions could occur, but I don't think one can say at this time, they will occur.

COLWELL: I am compelled to make a brief statement concerning low nutrient concentrations in seawater. It is obvious that suspended particulate matter in seawater results in different concentrations of nutrients in the water column. Large concentrations can occur in microniches, an important consideration. For toxic chemicals and their effect on the environment, we must be concerned that we may be disrupting the community structure of the microbial populations. For example, fuel oil, or refined oil, will selectively inhabit or perhaps kill organisms that are capable of carrying out certain ecologically important activities such as cellulose decomposition or starch hydrolysis, whatever that effect may be on the total ecosystem. Microorganisms in the environment carry out ecological functions. Effects of toxic chemicals considered to date have not been thought about in this respect. What effects are there on the natural populations and, therefore, on the ecosystem? I think that is an aspect of microbial ecology we must consider.

TRANSFORMATION IN AQUATIC ENVIRONMENTS

Chairperson, Carol Litchfield

NUTRIENT LIMITATION

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ABSTRACT

Substances which are degradable in the laboratory can become apparently recalcitrant in the natural environment because of the lack of a necessary nutrient. The physical state of the nutrient, such as dissolved, biphasic or particulate, can govern the rate of biologically induced change in the system. The commonest nutrient deficiency in the oceans is a lack of nitrogen and less often phosphorus. Hence, to degrade organic material, microorganisms must be able to mobilize the nutrients efficiently, and this will depend upon the biochemical constants of the enzymes involved. These points are illustrated using crude oil as an example, both of molecules which are recalcitrant because of their chemical structure and also because of the low concentrations of nitrogen in sea water.

In discussing the problem of pollutants in the marine environment, and the factors that govern their disappearance by biological action, we are attempting to explore the growing point between some aspects of marine microbiology and marine chemistry. The area is hardly overstocked with facts. Certainly, there are an increasing number of papers about marine organic chemistry (7), and considerable work was done a few years ago on the effects of substrate limitation on the growth of pure cultures in a chemostat. The effect of starving bacteria also has been studied by many workers and their findings have been reviewed by Dawes (5). Unfortunately, most of this work is only obliquely related to the task of this workshop. As so often in the past, it is not the production of bacterial biomass that concerns us primarily, though this is clearly relevant, but the removal of foreign chemicals which have been placed in the marine environment by human activity, and whose concentration and chemistry can be changed by nonbiological phenomena such as wind and wave mixing, dilution, and photochemical reaction.

In fact, there is still comparatively little information about the rates of destruction of organic substances in the sea, and most of the available information deals with the fate of small, but ecologically significant,

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molecules. Unhappily, too many of the papers dealing with the fate of pollutants describe experiments carried out under conditions very different from those found in the sea. Generally, the tendency has been to maximize the rates of decay, leading to over-optimistic forecasts of the duration of the pollutant in the oceans. However, we do know that some organics, both natural and human artifacts, are only slowly broken down. Whatever may be the final definition of persistence or recalcitrance to emerge from this workshop, it seems that there are at least three possible reasons why a carbon substrate may not be degraded in the marine environment. These are:

1) There are some substances whose molecules are so strongly constructed that an excessive amount of energy is required to change them into a form that can enter the biochemical mechanisms of the microbial cell. Because of this large expenditure of energy, the breakdown rate is necessarily slow.

2) There is another set of circumstances in which the microbial population does not have the ability to destroy the molecule. In other words, the information as to how to make the appropriate enzymes does not exist within the gene pool of the population. Hence, attack is impossible.

3) Finally, there are those instances where, although breakdown is energetically feasible and degradation pathways are available, oxidation is prevented by environmental conditions. Such conditions include unsuitable temperatures, lack of suitable electron donors, or a paucity of nutrients. All three reasons for recalcitrance can co-exist at one time, but in this paper we are concerned only with the third. In certain instances, the missing moiety may be a growth factor or a metal. For this reason, yeast extract is often added to media to isolate heterotrophs. Occasionally, growth factor dependency leads to mutualism (22). Again, Litchfield and Floodgate (14) found sediment organisms which required amino acids while Dibble and Bartha (6) found that soluble iron stimulated the removal of crude oil. Nevertheless since most natural populations, as a whole, are not likely to be nutritionally exacting, the commonest reason for the nondevelopment of microorganisms in an otherwise generally favorable environment is most likely to be an insufficiency of a major nutrient. In the sea, nitrogen and phosphorus are the most probable candidates; indeed, the commonest constraint on bacterial growth in the sea is a dearth of available nitrogen. Phosphorus is less likely to be the limiting factor since it appears to be rapidly recycled (18). However, Le Petit and N'Guyen (13) have observed that the optimum concentration for bacterial growth on hydrocarbons in natural waters depends on the ionic strength. The concentrations for maximum growth ranged between 2×10^{-4} M to 8×10^{-4} M phosphate in waters of salinity close to that of oceanic water and between 1.5×10^{-3} M to 3×10^{-3} M phosphate where the natural water receives a large input of low salinity water. Excessive amount of phosphate also inhibited bacterial growth.

Perhaps the best known example of nutritionally induced recalcitrance in the sea is that of oil degradation. This has been demonstrated by many workers but is shown here (Fig. 1) in the well known example provided by Atlas and Bartha (1). Presumably, nitrogen limitation acts by either preventing the growth of sufficient biomass to deal with the substrate, or by preventing the organisms that are there from being maximally active. It is generally accepted that a high bacterial count does not necessarily imply a high degree of activity and vice versa. Further, it must be assumed that the organic matter is not able itself to supply nitrogen, so that this form of limitation is confined to those substrates that are low in available nitrogen or from which nitrogen is absent. Therefore, the substrates involved are largely carbohydrates and their polymers such as cellulose and starch, lipids (including steroids), hydrocarbons (including spilled oil), some phenolics, and a number of synthetic pollutants.

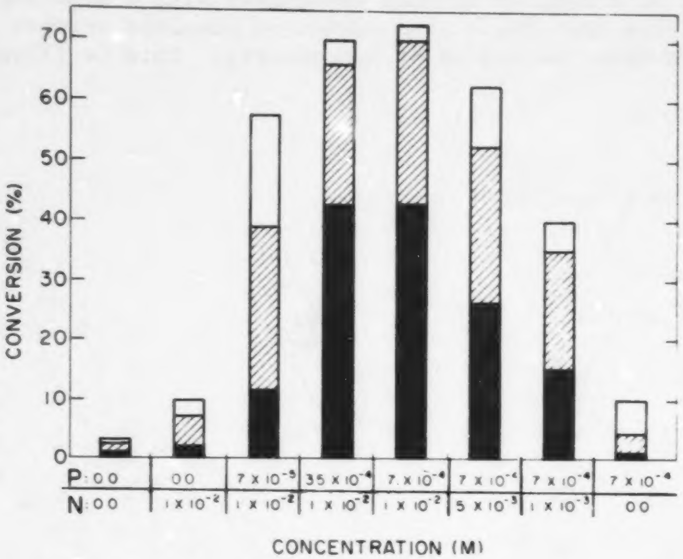


Figure 1. Conversion of petroleum in sea water supplemented by various concentrations of Na_2HPO_4 and KNO_3 during 18 days of incubation. Solid portions of the histograms represent mineralization percentage as measured by CO_2 evolution. The upper limits of the cross hatched and empty portions of the histograms represent biodegradation percentages as measured by gas chromatography and by residual weight, respectively (from Atlas and Bartha [1]). Normal sea water concentrations of nitrogen and phosphorus are below any of the additions by several orders of magnitude.

It is well established that various bacteria, especially certain species of *Desulfovibrio* and Clostridia, are able to fix nitrogen, the ATP required coming from the oxidation of organic matter (2). Hexadecane has been reported as providing a carbon and energy source for nitrogen fixing (19) soil bacteria. However, it is generally concluded that bacterial nitrogen fixation is an insignificant part of the annual nitrogen input to the seas (2).

It should also be remembered that the available organic matter will be very dilute and variable in concentration. Hence, there are advantages in thinking, not only in terms of the concentration of the nitrogen and carbon, but also of the carbon to nitrogen ratio. However, the significance of this ratio is complicated by the fact that the dissolved organic matter is not the only degradable form of organic carbon in the marine environment. There is the particulate matter as well, and whereas the greater part of the dissolved organic matter is known to be in a recalcitrant form such as "gelbstoff," much of the particulates are composed of polymers such as starch, cellulose, etc., that are intermediate in their degradation rates. Clearly, in the example of such particulate organic carbon, or of oil, either floating or on the surface or in finely divided form, we are dealing with a biphasic system in which the bacteria are either attached, or closely associated with a separate carbon-rich phase. It follows that there is a series of possible systems in which the C/N ratio varies with the degree of homogeneity. This is illustrated in Figure 2.

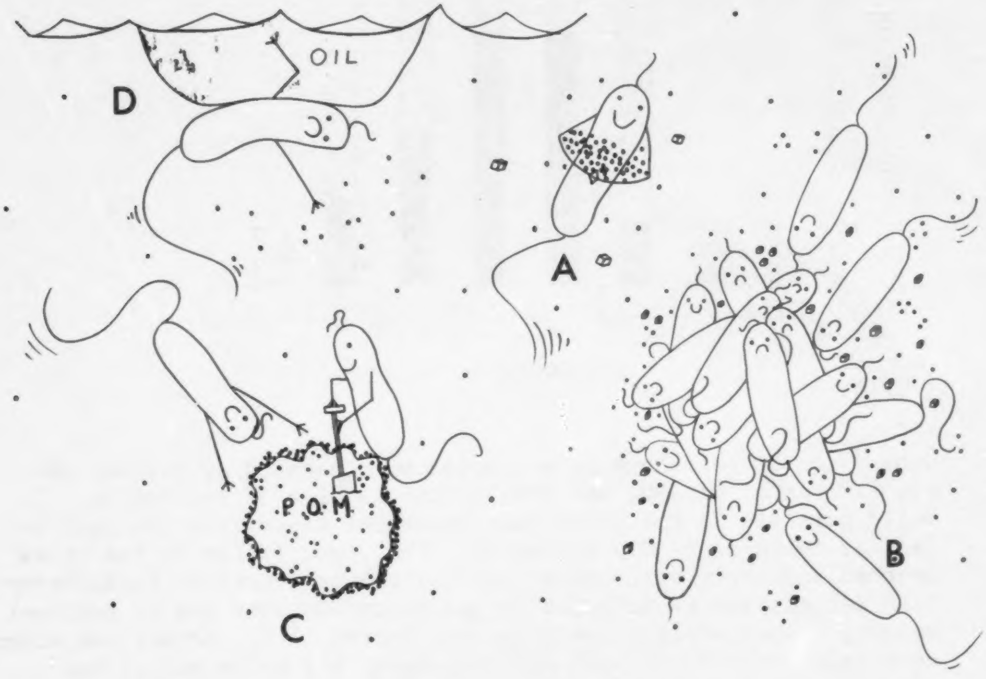


Figure 2. Effect of homogeneity of environment on carbon/nitrogen ratio. ● indicates available nitrogen; □ indicates available carbon source. For discussion see text.

Organism A is an authochthanous bacterium, that is, its resources are scarce but its ability to harvest these resources is high. In contrast, the organisms at B have plentiful supplies in terms of available carbon and nitrogen. This has led to overgrowth, with consequent deterioration of the culture. The organisms on particulate matter at C have solid phase organic carbon on which to adhere; inorganic nitrogen has sorbed onto the detritus, setting up a favorable C/N ratio. The hydrocarbonoclastic bacterium at D is closely associated with the oil phase but like the organism at A, has to scavenge nitrogen from the aqueous phase. A rather different view of the relationship between the oil phase and hydrocarbonoclastic bacteria has been proposed by Velankar and his colleagues (22). They suggested that surface active material, possibly formed by the bacteria themselves, remove miscelles from an oil droplet, the miscelles being about one tenth of the size of a bacterium. The miscelles "recruit" nutrient ions around themselves and the whole is transported across to the bacterium which receives a packet of carbon, nitrogen and phosphorus. If this model is true, then the ability of the miscelle to scavenge nitrogen could be a limiting factor.

The variations in concentration of nutrients between geographical areas and with time will have a bearing on the biodegradation rate, particularly when it is remembered that the most pronounced changes occur in the shallow coastal waters, which are the most productive as well as the most likely to receive polluting wastes. Detailed information of the amounts of nitrogen in these environments can be found in any major marine chemistry text (e.g., 18 and 20), but for the sake of convenience it is worth recalling a few salient facts. The concentration of organic nitrogen, both dissolved and particulate, varies from 5 to 10 μM-N. The inorganic nitrogen is found in several oxidation states, of which nitrate is the most important. Concentrations of up to 600 μg nitrate-N l^{-1} are found, while nitrite and ammonia are present at up to approximately one tenth of this amount. Temperate surface oceanic water contains around 10-15 μg nitrate-N l^{-1}. In tropical water this might fall below 1 μg nitrate-N l^{-1}. The amount increases with depth of the water column up to about 1000 m where it reaches a value between 300 and 600 μg nitrate-N^{-1}. The concentration remains at about this value to the bottom. From the degradative point of view, the comparatively high concentrations in estuaries and coastal waters will assist decomposition but once the recalcitrant material has escaped to the oceanic water, the lack of nitrogen is likely to further delay oxidation, particularly if the pollutant stays near the surface. There are also important seasonal variations to consider. Much of this is due to the competitive effects of the algae which lock up large amounts of nitrogen in the spring and autumn blooms. There are other variations caused by discontinuities such as thermoclines and frontal systems, and sometimes a patchiness of no apparent origin. A recalcitrant material therefore might meet a variety of nitrogen regimes before it finally disappears.

Although there is very little evidence as to the fate and function of nutrients in marine microbial degradation, our work on oil in sea water using a fixed amount of oil and varying amounts of added nutrients led to development of the idea of "nitrogen demand." This is defined as the amount of nitrogen required in the breakdown of 1 mg of oil, at least insofar as it was changed over an experimental period of nine to twelve months (8, 9, 10). The idea differs in several important aspects from the Biochemical Oxygen Demand, the most important difference being that the oxygen of the BOD is directly involved in the chemistry of the breakdown whereas the nitrogen is not. Hence, a stereometric relationship exists at least theoretically between BOD and the oxidation of the substrate. No such relationship exists with the nitrogen demand which is related only to the degradative ability of the system under consideration. (There is, of course, an exception in the cast of nitrate dissimilation where the nitrate is part of the electron acceptor mechanisms.) Using a variety of nitrogen regimes that fell within the variations that are known to occur in the Irish Sea, Gibbs (8) showed a linear relationship between the rate of oxygen uptake and the rate of nitrogen uptake. He found a regression equation of

$$O_2 \text{ uptake mg week}^{-1} = 0.72 + 0.83 \text{ (nitrogen uptake µmol week}^{-1})$$

If it is assumed that the theoretical oxygen demand for crude oil is 3.5 g oil oxidized per gram oxygen, it is possible to calculate the amount of nitrogen assimilated in association with the disappearance of 1 mg oil. Under the experimental conditions used, at 14°C and with Kuwait crude oil as the substrate, the value works out at. 4 µ moles N mg^{-1} oil. If crude oil is assumed to be 90% carbon, the ratio of carbon oxidized to nitrogen assimilated, calculated on a molar basis, is 18:8 for summer temperature and 6:8 in winter. Furthermore, using this data and making some assumptions as to the rate of nitrogen regeneration, Gibbs (8, 9) calculated that one cubic meter of Irish sea water would provide sufficient nitrogen to degrade 30 g oil in summer $year^{-1}$ and 11 g oil $year^{-1}$ at winter temperature.

A second set of data was obtained using a continuous flow method in which a stream of water was passed through a tube whose inner surface was coated with oil (10). Again assuming that oxygen remained in excess, nitrogen uptake rates were the limiting factor. For the apparatus running at 14°C the regression analysis generated the equation

$$\text{Oxygen uptake mg hr}^{-1} = 0.005 + 0.33 \text{ (N supply µ mole hr}^{-1})$$

This was felt to be satisfactorily close to that for the respirometry experiment.

In a third experimental design (11), a gravel column was used as a matrix for the standing oil phase, and sea water containing different concentrations of nutrient at several percolation rates was passed down it. Again, providing low concentrations of oxygen were avoided by a high flow rate, nitrate addition rates were the limiting factor. The regression equation under these conditions was

$$\text{Oxygen uptake mg hr}^{-1} = 0.12 + 0.23 \text{ (nitrate supply µ moles hr}^{-1})$$

Unfortunately, none of these experimental designs allow the worker to monitor the biomass, numbers or types of microorganism without terminating the experiment. Nevertheless, it was possible to obtain some information by examining the effluent water. These data suggest that the bacteria developed rapidly in the early days to a value of around 10^5 bacteria ml^{-1} as shown on normal heterotrophic media, and subsequently stayed more or less steady. This would indicate that the nitrogen that was being continuously added was not being used to increase bacterial numbers or biomass, but only to replace dead cells or possibly was stored in the amino acid pool.

The inorganic nitrogen uptake was determined by difference from the known added amounts and that found in the effluent water. This is illustrated in Figure 3 from the respirometry experiments using data from a flask receiving 2 µmol N/$week^{-1}$ as sodium nitrate and 0.2 µmol P/$week^{-1}$ as sodium dihydrogen phosphate (8). Although the oxidation proceeded at a more or less steady rate for several months, the amount of nitrogen in the flask was close to zero whatever the rate of nitrate addition. The disappearance of the nitrogen clearly poses an interesting series of questions. Figure 4 shows the change in the nitrogen concentration during one week (No. 17) of the flask receiving the highest rate of nutrient addition. It is clear that the inorganic nitrogen is almost completely assimilated by the microbial population within three days.

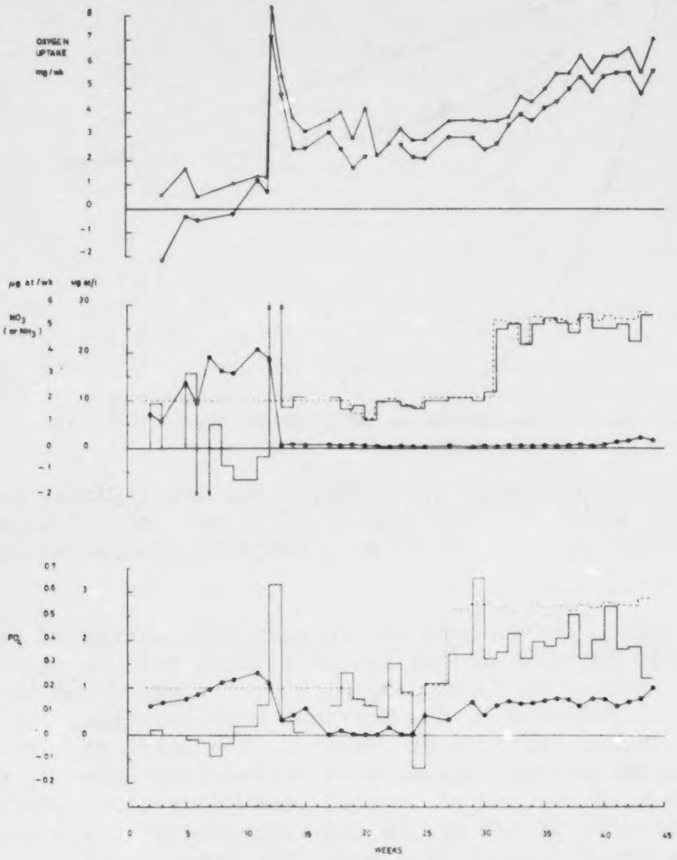


Figure 3. From Gibbs (8). Oil added at week 12. ▲ = gross rate of oxygen uptake; ▼ = rate of oxygen uptake less control; ---- = nutrient added; —— = nutrient utilized; ■ = nitrate concentration; ● = phosphate concentration.

Unfortunately, the result was not exactly reproducible. When the experiment was repeated using another batch of aged sea water, the nitrate concentrations were rapidly reduced in a few days but not to the same degree as in the first instance. The difference is believed to be due to a change in the microbial population in the area of the Irish Sea from which both samples were taken.

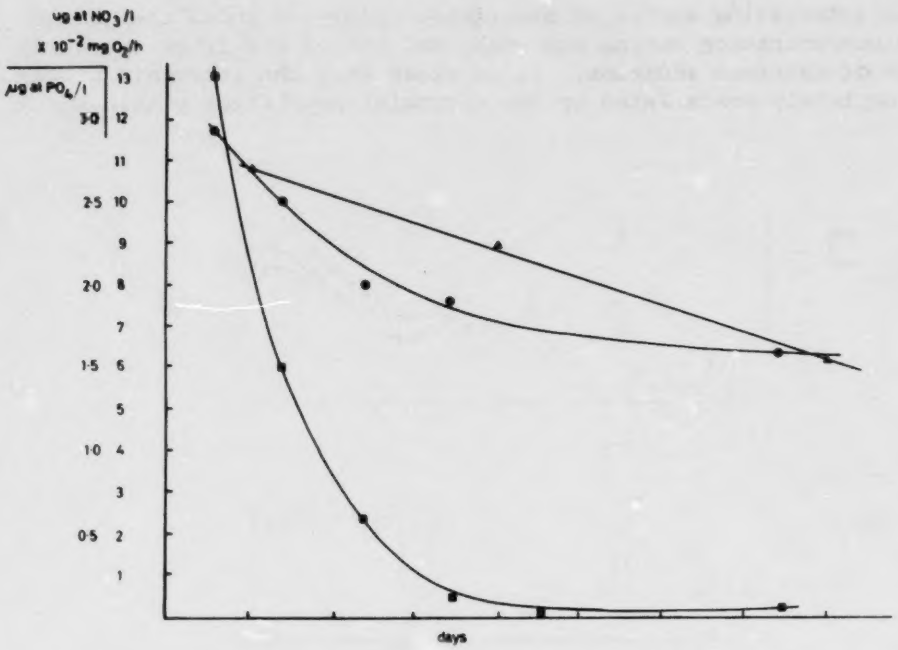
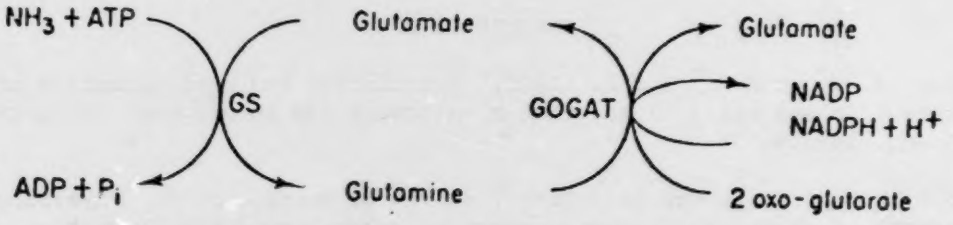


Figure 4. From Gibbs (8). [illegible] 7 in detail for respirometer receiving 2 µmol N week^{-1} and 0.2 µmol week^{-1}. ▲= rate of oxygen uptake; ■ = nitrate concentration; ● = phosphate concentration.

In an interesting, and to some extent parallel, series of papers Parks noted a relationship between utilization of nitrate and the oxidation of cellulose by several species of fungi (15, 16, 17). It was found that the rate of cellulose removal is affected by the nitrogen concentration, and that different species show various patterns of removal. The ratio of carbon removed from the substrate to nitrogen assimilated varied from below 10 to around 48 depending upon the chemical and biological conditions (15). Park (17) also emphasized the ambiguity of C/N ratios and suggested that the carbon/mineral ratios should be expressed in three forms. The provisional ratio is the proportion of the nutrient elements that are available in any particular environment. It is suggested that this ratio will be a factor in determining which of several possible decomposers in the vicinity of the substrate will become the dominant organism. The second ratio suggested is the assimilation ratio, which is the amount of carbon in the decomposer biomass per unit of nutrient assimilated, and thirdly, the decomposition ratio which is the amount of carbon oxidized per unit mineral element assimilated. This third ratio is clearly close to the nitrogen demand which we have suggested for oil decomposition in the sea. It should be remembered that although nitrogen demand or decomposer ratio is useful for the purpose for which it was originally intended, i.e., as a means of estimating the acceptable loading of a recalcitrant pollutant in a particular sea area, it is limited in that there is no certainty that the organic material is mineralized to carbon dioxide. If the carbon is only partially oxidized, then the nitrogen demand will increase before complete mineralization is achieved.

The question of what happens to the nitrogen and how it is assimilated by the organisms is obviously of interest at this point. Nitrate, the form in greatest supply in the sea, first has to be reduced to ammonia by nitrate and nitrite reductases. Syrett (21) has calculated that the reduction via nitrite to ammonia involves a free energy change of over 70 kcal mol^{-1} so it is not surprising that ammonia is the preferred source. For one pseudomonad isolate, Brown and his colleagues (3) found that the uptake of nitrate ceased if the ammonia concentration went above 1 mM, but at lower concentrations both sources were used simultaneously. The necessity to reduce nitrate means that the rate of uptake and reduction of nitrate is a physiological limitation on the rate of nitrogen availability even though the supply of inorganic nitrogen may be adequate. However, the saturation constants for nitrate and ammonia uptake were 0.26 mM, and for nitrate reductase 0.29 mM. These figures represent a fair degree of scavenging ability for low concentrations of nitrate.

The mechanism for ammonia assimilation also has been extensively studied (2, 4). There are two known major routes. First there is the classical glutamate dehydrogenase route whereby 2-oxo-glutarate reacts with ammonia to give glutamic acid. This system has a high K_m value of around 10-22 mM so that this is not a good mechanism for scavenging ammonia and will only be important when the ammonia concentration is his; thus, it is rarely important in the sea. However, the mechanism is independent of the presence of ATP. The second route (Fig. 5) requires ATP. Here assimilation is by way of glutamine synthetase (GS) and glutamate synthase (GOGAT•glutamine (amide); 2-oxo-glutamate amino transferase). The K_m of glutamine synthetase is about 0.25 mM so that it will mobilize ammonia at low concentrations. Therefore, both major pathways for the reduction of nitrate and the assimilation of ammonia in the marine environment necessitate expenditure of energy. It is possible that a bacterium which has lived under the starvation conditions, which exist over the greater part of the world's oceans, may not be able to readily assimilate the nitrogen that it requires and thus be unable to attack the polluting material. Hence, the previous history of the organism, as well as its genetic composition, may determine whether or not it will develop on a substrate.



Glutamate synthesis in ammonia-limited *Aerobacter aerogenes.*

Figure 5. The glutamine synthetase-glutamate synthase (GS GOGAT) pathway of nitrogen assimilation.

Once the ammonia has been assimilated into glutamate, it may form part of the amino acid pool of the bacterium and is then available for protein synthesis among other functions. However, a number of environmental factors affect the size of the amino acid pool. It is known (12) that the pool size of nitrogen-limited pseudomonads in a chemostat increased three-fold as salinity increased from 0.2 M to 0.5 M. The pool size also depended upon whether the culture was carbon or nitrogen-limited. With carbon limitation, the pool size was 3.9 mg liter^{-1} on 0.2 M sodium chloride and 14 mg liter^{-1} at 0.5 M sodium chloride. When the conditions were nitrogen-limiting the figures were much smaller at 0.4 mg liter^{-1} at low salinity, to 2.4 mg liter^{-1} at the higher salinity value.

In summary, the limitation of nutrients on degradation of pollutants in the marine environment appear to have a number of general effects. First, because the organisms which can degrade the substrate are in competition, the concentration of nutrients may determine which species or strain is likely to become dominant. The strain which is most effective in mobilizing the nitrogen or other nutrient in short supply will have an advantage. Second, the rate at which the nutrients are regenerated will govern the rate of degradation as this will regulate their concentration and so limit the growth of biomass, or the synthesis of enzyme, or both. Third, the characteristics of the assimilation pathways of the nutrients and the availability of energy within the cell can be the limiting factor even when the environmental concentration of the nutrient is not. Finally, the fate of the nutrient within the cell may determine whether it is used in the degradation of the pollutant in question or whether it is diverted to some other function. In all these areas, further research is needed, particularly directed to what happens in the natural environment rather than in the laboratory alone.

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LITERATURE CITED

1. Atlas, R. M., and R. Bartha. 1972. Degradation and mineralization of petroleum in sea water; limitation by nitrogen and phosphorus. Biotech. Bioeng. 14:309-318.

2. Brown, C. M., D. S. McDonald-Brown, and J. L. Meers. 1974. Physiological aspects of microbial inorganic nitrogen metabolism. Adv. Microbial Physiol. 11:1-52.

3. Brown, C. M., D. S. McDonald-Brown, and S. O. Stanley. 1975. The inorganic nitrogen metabolism in marine bacteria; nitrate uptake and reduction in a marine pseudomonad. Mar. Biol. 31:7-13.

4. Brown, C. M., and B. Johnson. 1976. Inorganic nitrogen assimilation in aquatic microorganisms. Adv. Aquatic Microbiol. 1:1-113.

5. Dawes, E. A. 1976. Endogenous metabolism and survival of starved prokaryotes. Symp. Soc. Gen. Microbiol. 26:19-53.

6. Dibble, J. T., and R. Bartha. 1976. Effects of iron on the biodegradation of petroleum in seawater. Appl. Environ. Microbiol. 31:544-550.

7. Ehrhardt, M. 1977. Organic substances in the sea. Mar. Chem. 5:307-316.

8. Gibbs, G. F. 1975. Quantitative studies in marine biodegradation of oil. I. Nutrient limitation at 14°C. Proc. Roy. Soc. Lond. Ser. B. 188:61-82.

9. Gibbs, C. F., K. B. Pugh, and A. R. Andrews. 1975. Quantitative studies in marine biodegradation of oil. II. Effects of temperature. Proc. Roy. Soc. Lond. Ser. B. 188:83-94.

10. Gibbs, C. F. 1977. Rate measurements and rate limiting factors in oil biodegradation in the marine environment. Rapp. P-v. Reun. Cons. Int. Explor. Mer. 171:129-138.

11. Gibbs, C. F., and S. J. Davis. 1976. The rate of microbial degradation of oil in a beach gravel column. Microbial Ecol. 3:55-64.

12. Herbert, R. A., C. M. Brown, and S. O. Stanley. 1977. Nitrogen assimilation in marine environments, pp. 161-177. *In* F. A. Skinner and J. M. Shewan, eds., Aquatic microbiology. Appl. Bact. Symp. Ser. No. 6, Academic Press, New York.

13. Le Petit, J., and M.-H. N'Guyen. 1976. Besoin en phosphore des bacteries metabolisant les hydrocarbures en mer. Can. J. Microbiol. 22:1364-1373.

14. Litchfield, C. D., and G. D. Floodgate. 1975. Biochemistry and microbioloty of some Irish Sea sediments. II. Bacteriological analysis. Mar. Biol. 30:97-103.

15. Park, D. 1976. Cellulose decomposition by Pythaceous fungus. Trans. Brit. Mycol. Soc. 66:65-70.

16. Park, D. 1976. Nitrogen level and cellulose decomposition by fungi. Int. Biodeterioration Bull. 12:95-99.

17. Park, D. 1976. Carbon and nitrogen levels as factors influencing fungal decomposers, pp. 41-59. *In* J. M. Anderson and A. Macfayden, eds., 17th Symp. Brit. Ecol. Soc. Blackwell Scientific Publ., Oxford.

18. Riley, J. P., and R. Chester. 1971. Introduction to marine chemistry. Academic Press, London.

19. Riviere, J., J. Oudot, J. Jonqueres, and G. Gatelier. 1974. Fixation d'azote atmospherique, par des bacteries utilisant l'hexadecane comme source de carbon et d'energie. Annals Agron. 25:633-644.

20. Spender, C. F. 1975. The micro-nutrient elements, pp. 245-295. *In* J. P. Riley and G. Skirrow, eds., Chemical oceanography, 2nd ed., vol. 2. Academic Press, London.

21. Syrett, P. J. 1954. Ammonia and nitrate assimilation by green algae. Symp. Soc. Gen. Microbiol. 4:355-427.

22. Velankar, S. K., S. M. Barnett, C. W. Houston, and H. R. Thompson. 1975. Microbial growth in hydrocarbons; some experimental results. Biotech. Bioeng. 17:241-251.

23. Zavarin, G. A., and A. N. Nozhevnikova. 1977. Aerobic carboxydobacteria. Microbial Ecol. 3:305-326.

NATURAL HETEROTROPHIC ACTIVITY IN ESTUARINE AND COASTAL WATERS

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ABSTRACT

Previous studies on natural heterotrophic activity have contributed substantially to our understanding of the role of the bacteria in natural waters. The research has demonstrated that low molecular weight organic compounds are present at concentrations of several micrograms per liter or less, and the existence of natural bacteria populations capable of uptake at these low substrate concentrations. These microbes account for rapid turnover and substantial mineralization of substrates. Some specificity is shown in the response of natural microbes to organic solutes, along with a considerable range in rates of uptake. This range is responsive to the degree of organic pollution, differences in temperature, and other factors less easy to assess, such as the physiological state of the bacteria.

Recent establishment of epifluorescence direct counting using Acridine Orange has provided a method that gives total counts of bacteria in natural waters. This method can be combined with radioisotope-based measures of heterotrophic activity to give a quantitative expression of the relationship between activity and numbers of heterotrophic bacteria, the "specific activity index." Major differences in specific activity were found in coastal and estuarine waters of northeastern Massachusetts. These differences follow changes in habitats; e.g., a decline with depth in the water column and with increasing distance from the coast. This approach, applied to enrichment studies, has revealed an interesting pattern of bacterial response to enrichment. Cell numbers increase linearly, while activity increases exponentially, as does the plate count. Based on the results of these studies and the work of others, an adaptive strategy of the natural heterotrophic bacteria can be postulated that involves reversible changes from functional dormancy to a high level of activity. Additional responses to enrichment, or pollution, include growth and multiplication, and certainly selection. The sequence of responses is discussed and related to the problems of measuring the response of a natural population of bacteria to any added substance.

INTRODUCTION

For the past fifteen years freshwater and marine microbial ecologists have been using radiolabelled organic solutes to probe the activities of heterotrophic bacteria in natural waters and sediments. These workers have employed methods designed to deal with microbial populations under conditions as close as possible to those in nature. The basic goal of this work has been to understand the activity of the heterotrophic bacteria in relation to the fundamental processes of energy flow and material cycling in aquatic ecosystems. Obviously, this information is essential for understanding of the potential role microbes might play in breakdown of organic pollutants. The first part of this paper is a presentation of the most significant results of previous studies of heterotrophic activity as they relate to the goal of this conference. Following this is presented some recent work on the relationships between microbial numbers and activity in estuaries and coastal waters.

I. HETEROTROPHIC BACTERIAL ACTIVITY--AN ASSESSMENT

A. DISSOLVED ORGANIC COMPOUNDS IN NATURAL WATERS

Research on heterotrophic bacterial activity has confirmed the information obtained from the better chemical analyses concerning the normal concentration range of low molecular weight organic compounds, i.e., below 200 molecular weight. This confirmation derives from a kinetic analysis of uptake at substrate concentrations in the range of 1-100 μg/liter (35,39). The analysis yields a parameter $(K_t + S_n)$, which is a combined value including natural substrate concentration (S_n), and a transport constant (K_t) indicative of the affinity of the microbial transport systems for the substrate in question. Measured $(K_t + S_n)$ values are useful in setting an upper limit on S_n, certainly giving an order of magnitude indicative of the natural substrate concentration. $(K_t + S_n)$ values have been reported for a number of substrates from a variety of aquatic systems, and have been compared with S_n measured chemically in a number of studies (3,6,9,20,41).

Essentially, the low molecular weight compounds are singly present at concentrations of several micrograms per liter or less, results that indicate higher concentrations are highly suspect. For example, the concentration of total dissolved free amino acids in estuarine and coastal waters, measured chemically, shows a range of 25 to 100 micrograms per liter (3,9). Even if the waters are polluted, the range is at most only double the normal level. Offshore and deeper waters indicate lower levels, from 2 to 10 micrograms per liter (3,33). This has led to the important understanding that in order to work with the natural bacteria, one must measure their activities relative to such low substrate concentrations.

B. HETEROTROPHIC USE OF DISSOLVED ORGANIC COMPOUNDS

Using simple radiolabelled organic compounds like glucose, glutamate and acetate, many workers have shown that heterotrophic microbes in eutrophic and mesotrophic waters are active in taking up and metabolizing substrates at concentrations of a few micrograms per liter. The uptake can be characterized as follows:

(1) Uptake shows a saturation type response to substrate concentration which can usually be analyzed with Michaelis-Menten kinetics and is best measured in a concentration range of one to 50 micrograms per liter. This clearly indicates that a most important adaptation for heterotrophic microbes is the possession of transport systems functional in this concentration range. Measurements of transport constants have confirmed this (14,28,39).

(2) The turnover of many low molecular weight compounds is rapid, from a few hours or less in summer conditions to several days or more in colder waters or in winter. Turnover is much more rapid in polluted and eutrophic waters than in oligotrophic waters. Obviously, to accurately assess the importance of a given compound to heterotrophs it is necessary to measure more than just its concentration. Two of the standard methods employed for measuring heterotrophic activity yield the natural turnover time (5,12,32,39), defined as the ratio of substrate concentration to rate of use.

(3) A substantial proportion of substrate taken up is respired or mineralized. The percent varies, depending on the substrate and other factors, from 1 or 2% to as much as 80% for some substrates like glycolic acid (12,19, 40). Most commonly, respiration is in the range of 25-50% of uptake. After the first few minutes, this percentage is usually quite constant. This process indicates the major pathway for nutrient regeneration from dissolved organic matter in natural waters (3,36).

(4) There has been some confusion in the past as to which organisms are responsible for the measured heterotrophic activity in natural waters. Wright and Hobbie (39) used a kinetic analysis of natural and cultured populations of bacteria and algae to conclude that most of the uptake detected at close to natural substrate concentrations was due to the bacteria. Algal uptake of organic compounds appeared to be measurable only at relatively high and unnatural substrate concentrations. Two recent lines of research have established beyond reasonable doubt that the bacteria are the primary agents of natural heterotrophic use of dissolved organic solutes. The use of Nuclepore filters, with their superior properties of acting like a true sieve, has shown that in coastal and open ocean waters, 90% of the measurable heterotrophic uptake is in the fraaction less than one micron in size (4). Where significant heterotrophic activity is associated with larger particles, there is evidence that bacteria on detritus are responsible for most of the activity (16). Autoradiography has confirmed these results, giving a qualitative indication that most of the heterotrophic activity of a natural plankton community is

associated with the smallest particles, that is, the bacteria (22).

(5) Some substrates are actively taken up and metabolized by a given microbial population, while others are not. Those substrates most frequently used by active heterotrophic populations are the simpler compounds, like glucose, acetate, lactate and most of the amino acids, with obvious entry into the major biochemical pathways (7,15,20,40). In nutrient-poor waters, however, little heterotrophic activity can be measured for any substrate, and the uptake usually does not follow Michaelis-Menten kinetics (29). Thus, specificity occurs in terms of preferences for a given compound, and it is probably linked to the specificity of transport systems possessed by the major bacteria species in a population. The lack of measurable activity toward even the simpler substrates in some waters is a matter that will be addressed subsequently.

(6) Rates of heterotrophic uptake and/or mineralization of specific substrates at close to natural substrate concentrations are expressed as either V_{max} (heterotrophic potential) values or actual flux rates (39,41). The latter rate requires an independent measurement of substrate concentration and hence is less commonly performed. The V_{max} values are derived from a kinetic analysis of uptake, and are an expression of the instantaneous flux of substrate use by a given population if their use is saturated with substrate (see 37 for a discussion on this method). Measured either way, the rates show a broad range of several orders of magnitude in heterotrophic activity. V_{max} values have been reported as high as several $\mu g \cdot liter^{-1}$ (2,7,9.41), and they can be found at least four orders of magnitude lower (1,15). The factors controlling this broad range include temperature, size and physiological state of the existing microbial population, rate of input of usable dissolved organic matter, grazing by protozoa, and probably others.

(7) Studies of heterotrophic activity have uncovered some important ecological relationships. For example, there is evidence of a close energy coupling between planktonic algae and bacteria, with the algae nourishing the bacteria by excretion of dissolved organic carbon (11,18,31). It seems certain that this is the major autochthonous source of energy for the natural bacteria. Most of the excreted organic carbon is readily taken up and mineralized, while a fraction (ca 25%) is relatively recalcitrant (18).

Some workers have applied heterotrophic activity measurements to the sediments (8,17). The results have revealed very high heterotrophic activity concentrated at the surface of the sediments. In shallow waters, this may exceed the activity of the entire water column.

C. RESPONSE OF NATURAL HETEROTROPHIC POPULATIONS TO ENRICHMENT AND POLLUTION

Several workers have tested the response of natural microbial populations to short-term enrichment with low molecular weight organic solutes (30,34). These tests have involved concentrations of 25 $\mu g\ liter^{-1}$ up to several mg $liter^{-1}$, and have been measured in bottles for 24 to 48 h. More detailed information will be given subsequently, but some general findings of these experiments include: (a) a fairly constant rate of use for several hours, higher for higher applied concentrations but definitely not proportional to applied concentration; (b) a marked, often exponential increase in uptake and mineralization of the test substrate by the end of the first day; (c) exhaustion of the substrate in two days, if it was added at concentrations up to 500 μg/liter. Unfortunately, most of these experiments have not involved simultaneous measurements of microbial population changes. They do, however, indicate that natural microbial populations can rapidly accommodate a 10- to 100-fold increase in substrate. This certainly corroborates the low ambient concentrations of organic solutes in natural waters. The response has been found to be more rapid in onshore and estuarine waters than in the open ocean.

A closely related observation concerns the response of natural microbial activity to organic pollution. Where natural waters are obviously polluted, heterotrophic activity is typically higher than similar unpolluted waters by one or two orders of magnitude (2,7). Furthermore, significant rates of metabolism of more complex pollutants can be demonstrated. For example, significant ^{14}C-hexadecane metabolism was found in Tokyo Bay, where oil pollution is obvious (26).

II. HETEROTROPHIC ACTIVITY AS A BACTERIAL POPULATION PARAMETER

For some time workers have been measuring heterotrophic activity without measuring the microbial population responsible for that activity, the main excuse being the lack of suitable methodology. With the recent establishment of the Acridine Orange Direct Count (AODC) using epifluorescence and Nuclepore filters, that excuse is no longer valid. Although there are still some alternatives in methodology, the AODC is well enough understood, and simple enough, so that any laboratory equipped to do heterotrophic activity measurements should also be measuring the bacteria with AODC (21). To quote Ralph Daley in a recent methods symposium: "With a reasonable amount of preliminary testing and attention to procedural detail, AO epifluorescence can provide rapid, precise quantitative data on bacterial numbers in all marine and freshwater environments. The use of alternative methods for this specific purpose is, therefore, no longer justifiable" (10).

Given this counting technique and the already established methods for measuring heterotrophic activity with radioisotopes, the next logical step is to relate heterotrophic activity to the bacterial population. I have proposed the term "specific activity index" to give a quantitative expression of the relationship between activity and numbers (38). Because there are three basic approaches to measuring heterotrophic activity with labelled organic solutes, three indices will be required (37).

Details of the three approaches are given by Wright (37), while the application of these approaches to specific activity measurements is found in Wright (38). Very briefly, the numerator of the given specific activity index is a measurable function of heterotrophic bacterial activity (e.g., turnover rate, V_{max}, direct uptake) and the denominator is the direct count of bacteria from the sample measured.

There are several current problems that this approach brings particularly well into focus. One of these is the discrepancy between the normal range of bacterial numbers and the range in heterotrophic activity. In my studies of the seasonal, horizontal and vertical variation in bacteria in estuaries and coastal water in northeastern Massachusetts, I have found a range of 0.3 to 6 x 10^6 bacteria/ml, slightly over one order of magnitude. Heterotrophic activity, however, can vary several orders of magnitude. In Figure 1 is presented the V_{max} specific activity index for glucose uptake at a depth of one meter from well up the Essex estuary along a horizontal transect to a station 14 km offshore in the Gulf of Maine. Figure 2 presents the same kind of data for a vertical profile at the 14 km offshore station. Specific activity declined two orders of magnitude from a peak at the estuary inlet to the 40 m depth waters. Clearly, no direct correlation between numbers and heterotrophic activity can be expected; this finding holds for other substrates and for different seasons in these temperate marine waters. However, the changes in specific activity do show a pattern, one that corresponds with a profound change in marine habitats, from a shallow, salt-marsh bordered tidal river to the offshore station 46 m in depth.

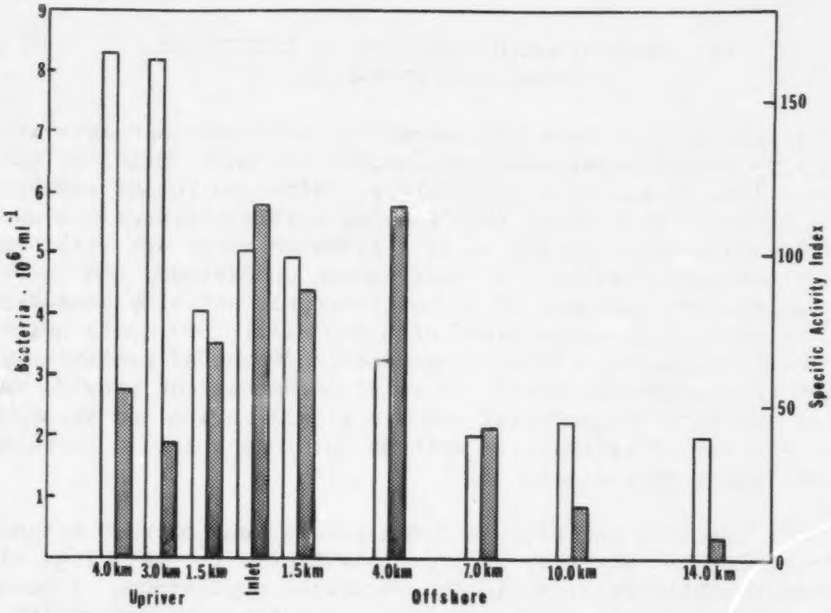

Figure 1. Bacteria numbers (clear) and V_{max} specific activity index (μg·cell^{-1}·hr^{-1}·10^{-12}) for glucose uptake (stippled) at one meter depth along a transect from within the Essex estuary to a station 14 km offshore, August 4, 1976.

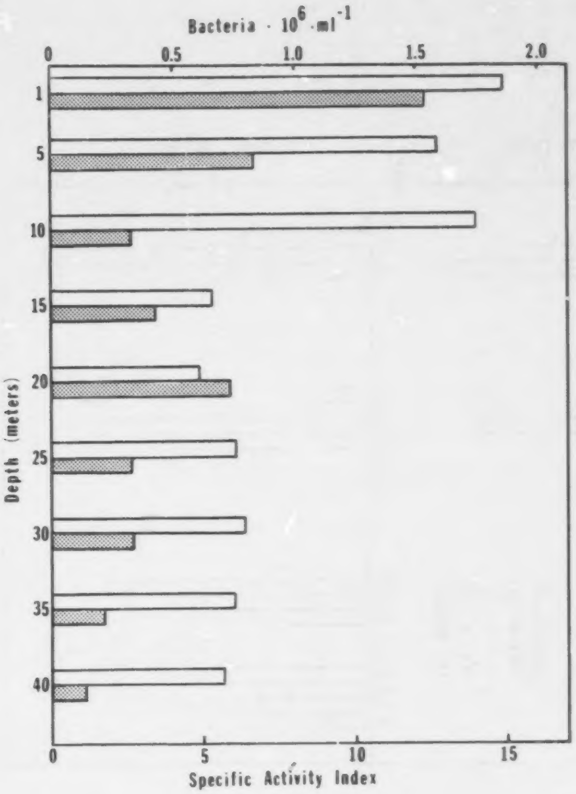

Figure 2. Bacteria numbers (clear) and V_{max} specific activity index ($\mu g \cdot cell^{-1} \cdot hr^{-1} \cdot 10^{-12}$) for glucose uptake (stippled) from a vertical profile at a station 14 km offshore in the Gulf of Maine, August 13, 1976.

Another current problem is the spectrum in sizes and shapes of the bacteria and the possibility that some of this spectrum may be indicative of changes in the physiological state of the bacteria, where the smallest bacteria are viewed as being starved forms with much lower physiological activity (27). Results from the Merrimack River, which is quite polluted in the freshwater part of the estuary, are shown in Figure 3. These data represent the separation of bacteria numbers and activity using Nuclepore filters. The upriver stations are in fresh water; the station 5 km from the inlet had a salinity of 7.3 ppt and the inlet station a salinity of 23.9 ppt. There is a pronounced shift in the size spectrum of bacteria from an apparently large pollution-related freshwater flora to a typical coastal marine flora dominated by the smaller bacteria. In the transition, specific activity declined abruptly. Because of the added parameters of freshwater to marine transition and pollution, this study does not give any clear answers to the question of starvation-related size and activity changes. This kind of approach, however, may be useful in solving the above question.

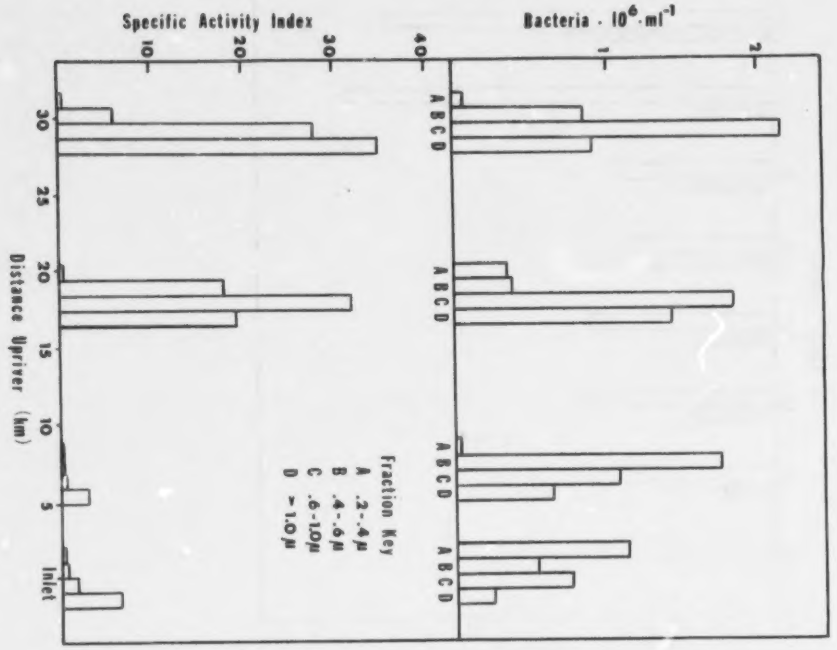

Figure 3. Bacteria numbers and turnover rate specific activity index (cell^{-1}·hr^{-1}·1·10^{-6}) from several stations along the Merrimack River estuary, one meter depth, November 21, 1977. Numbers and heterotrophic activity fractioned using Nuclepore filters of different pore sizes.

Some interesting things happen when a seawater sample is enclosed in a bottle and numbers and activity are followed over time. Results from a midwinter sample enriched with glucose and incubated at two temperatures (Fig. 4) illustrate two of the basic responses to enrichment mentioned earlier, i.e., an exponential increase in activity (even specific activity), and a basically linear increase in cell numbers. Similar enrichment experiments have been conducted using an additional indicator of bacterial activity. This is the method of Iturriaga and Rheinheimer (23), which gives a count of bacteria cells with active electron transport systems via the reduction of a tetrazolium salt (INT) to a red colored formazan. Figures 5 and 6 show the results of an experiment where 500 µg·liter^{-1} glutamate was added to the enriched sample. Specific activity is plotted against INT counts in Figure 7. These figures indicate that the incubation and enrichment conditions stimulate a substantial increase in bacterial activity accompanying a much lower increase in bacterial numbers. Some of the increase in specific activity is probably caused by an increase in size which occurs (Fig. 8).

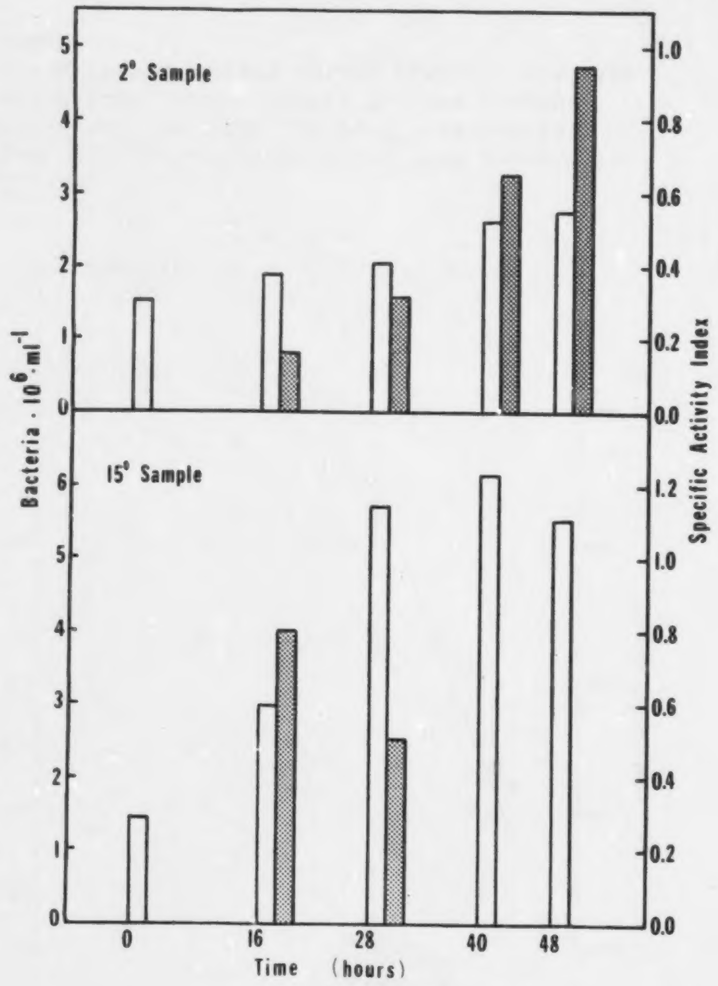

Figure 4. Bacteria numbers (clear) and direct uptake specific activity index (µg $cell^{-1} \cdot hr^{-1} \cdot 10^{-12}$) glucose uptake (stippled) in Essex estuary water enriched with 100 $\mu g \cdot l^{-1}$ (2C incubation) or 300 $\mu g \cdot l^{-1}$ (15C incubation) glucose.

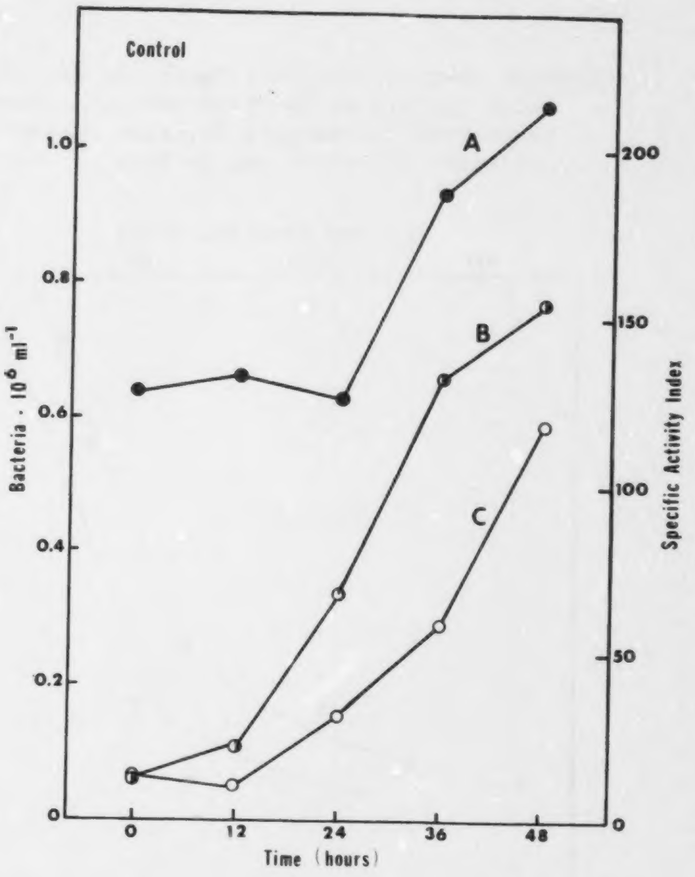

Figure 5. Essex estuary water (salinity 25.9 ppt, temperature -2°C) from January 31, 1978, incubated at 2°C for 48 h, unenriched. A = direct counts (AODC); B = bacteria with active electron transport (INT counts); C = glutamate turnover rate specific activity index ($cell^{-1} \cdot hr^{-1} \cdot l \cdot 10^{-6}$).

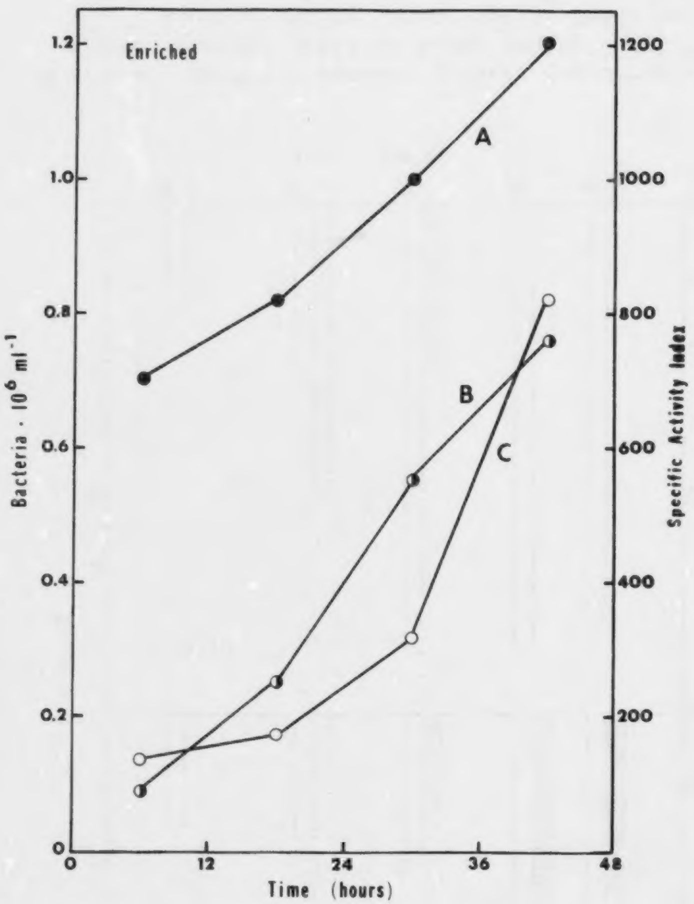

Figure 6. Essex estuary water from January 31, 1978, enriched with 500 $\mu g \cdot l^{-1}$ glutamate. A = direct counts (AODC); B = INT counts; C = glutamate direct uptake specific activity index.

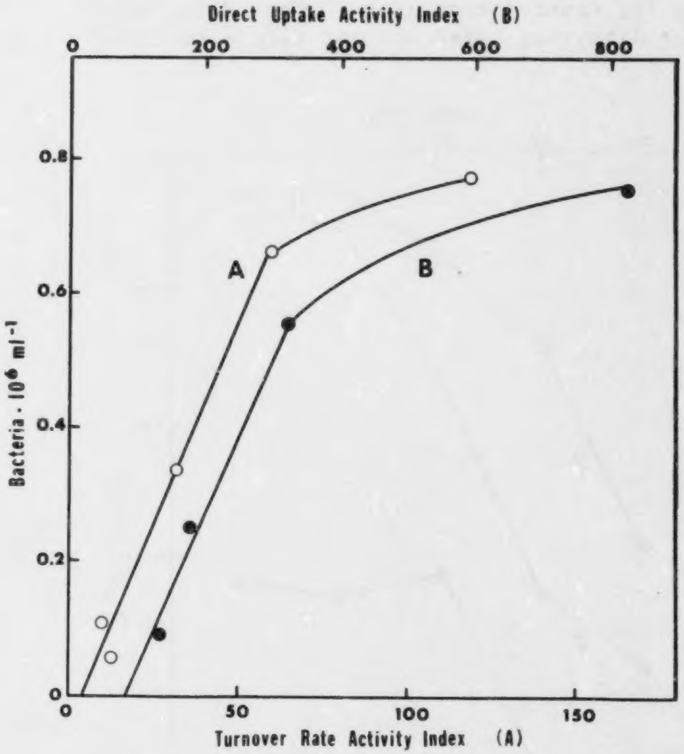

Figure 7. Plot of INT counts of bacteria vs specific activity indices for unenriched sample (A) and enriched sample (B) from January 31, 1978, sample from Essex estuary.

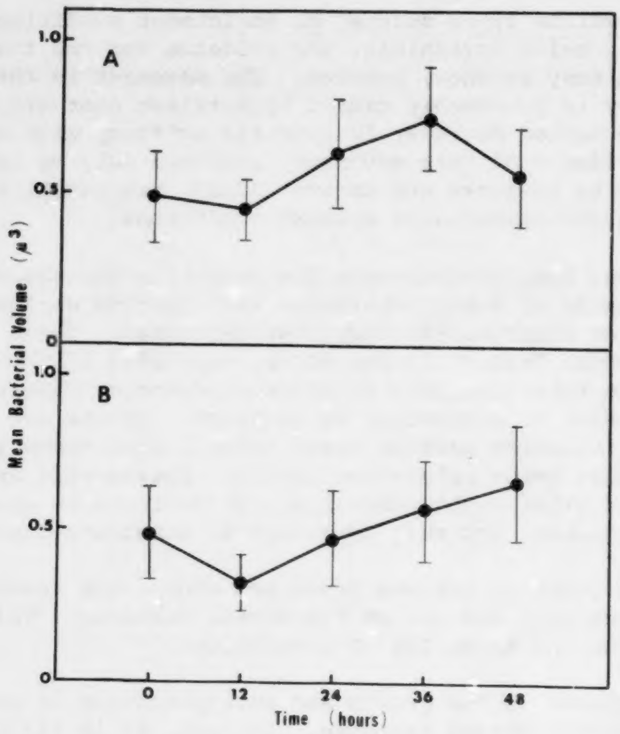

Figure 8. Mean bacterial volume (μ^3) measured microscopically fron enrichment experiment of January 31, 1978. A = control, B = enriched. Brackets indicate limits of mean ± 2 x $S.E._{\bar{x}}$.

It is instructive to summarize the bacterial responses induced by enclosure of a natural sample in a bottle, with or without specific enrichment. These are: (1) a linear, often delayed increase in direct counts; (2) an increase in specific (meaning per cell) heterotrophic activity, delayed at first but then exponential; (3) an increase in the percent bacteria showing active electron transport systems, at first rapid but eventually leveling off; (4) not shown by this experiment but repeatedly obtained when measured, an exponential increase in colony-forming units as measured by plate counts; (5) a slow increase in the mean volume of the bacteria. Based on these responses, a tentative analysis would suggest a basic adaptive strategy of the heterotrophic bacteria in natural waters. This strategy has been suggested in part by others (27). A natural heterotrophic population is made up of a spectrum of cells with a range of sizes and activity. Most of them are probably on the "down" side of functional activity, in a more or less dormant state (27). Given favorable conditions--enrichment, enclosure in a bottle--many, if not most, come out of dormancy. This may mean a number of things not well understood for natural bacteria--the energizing of membranes, the synthesis of permeases and internal enzymes. Whatever the causes, the changes can account for remarkable increases in heterotrophic activity without significant increases in numbers or selection of specific types adapted to enrichment conditions. This strategy is made complete by being reversible, the evidence for the reversal of the changes being less easy to show, however. The movement in the direction of functional dormancy is presumably caused by nutrient starvation. Figures 1 and 2, showing the marked decrease in specific activity with distance offshore and depth, give evidence of this movement, although only by inference. The important research by Novitsky and Morita (24,25) has established a similar reversible pattern for bacteria in culture conditions.

Practically any imaginable system for measuring the response of natural bacteria to pollutants or toxic substances will involve enclosure of a natural sample, and thus the adaptive strategy just described. There are at least two other kinds of changes that will also occur, depending on the amount of time of incubation. The first one is a function of transport kinetics, and is concentration dependent. No adaptation is required. Uptake and metabolism of a substrate will be increased because under natural conditions substrate concentration was somewhere below saturation levels. Uptake will approach and perhaps exceed V_{max} if other mechanisms--i.e., diffusion--are also present. This is a reversible response, entirely dependent on substrate concentrations.

The second response is the one described above--the reversible changes involved in movement into and out of functional dormancy. This response becomes evident during the first day of incubation.

The third response is the growth and multiplication of many of the bacteria successful in the second response. In fact, it is difficult to separate these two responses, because they are outcomes of the same physiological changes in the bacteria. Perhaps the best way to do this is to use cold temperature systems to expand the responses in time. After two or three days, the most available naturally occurring substrate is exhausted in the enclosed sample, as is the added substrate if it was readily metabolizable and added in the < 1 mg liter^{-1} concentration range. If higher concentrations were added, and if incubation is extended, a severe selection of bacteria will occur and continued growth will be a response to the high concentrations and the other enclosure conditions. By this time, the system is rather far removed from a natural population, and the results have increasingly less applicability to natural systems. They also have increasingly less predictive power and must be interpreted with great caution. Unfortunately, there may not be any reasonable alternatives to enclosing and incubating samples. When this is done, however, it is highly advisable to measure the population as well as its response.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Albright, L. J. 1977. Heterotrophic bacterial dynamics in the Lower Fraser River, its estuary and Georgia Strait, British Columbia, Canada. Mar. Biol. 39:203-211.

2. Allen, H. L. 1969. Chemo-organotrophic utilization of dissolved organic compounds by planktic algae and bacteria in a pond. Int. Rev. Ges. Hydrobiol. 54:1-33.

3. Andrews, P., and P. J. LeB. Williams. 1971. Heterotrophic utilization of dissolved organic compounds in the sea. III. Measurement of the oxidation rates and concentrations of glucose and amino acids in sea water. J. Mar. Biol. Assn. U.K. 51:111-125.

4. Azam, F., and R. E. Hodson. 1977. Size distribution and activity of marine microheterotrophs. Limnol. Oceanogr. 22:492-501.

5. Azam, F., and O. Holm-Hansen. 1973. Use of tritiated substrates in the study of heterotrophy in sea water. Mar. Biol. 23:191-196.

6. Burnison, B. K., and R. Y. Morita. 1974. Heterotrophic potential for amino acid uptake in a naturally eutrophic lake. Appl. Microbiol. 27:488-495.

7. Carney, J. F., and R. R. Colwell. 1976. Heterotrophic utilization of glucose and glutamate in an estuary: effect of season and nutrient load. Appl. Environ. Microbiol. 31:227-233.

8. Christian, R. R., and J. R. Hall. 1977. Experimental trends in sediment microbial heterotrophy: Radioisotopic techniques and analysis, p. 67-88. *In* B. Coull (ed.), Ecology of marine benthos. University of South Carolina.

9. Crawford, C. C., J. E. Hobbie, and K. L. Webb. 1974. The utilization of dissolved free amino acids by estuarine microorganisms. Ecology 55:551-563.

10. Daley, R. J. Direct epifluorescence enumeration of native aquatic bacteria: uses, limitations and comparative accuracy. Proc. ASTM Symposium, Native Aquatic Bacteria; Enumeration, Activity and Ecology, June 25, 1977, Minneapolis (in press).

11. Derenbach, J. B., and P. J. LeB. Williams. 1974. Autotrophic and bacterial production: fractionation of plankton populations by differential filtration of samples from the English Channel. Mar. Biol. 25:263-269.

12. Gocke, K. 1976. Respiration von gelösten organischen Verbindungen durch naturliche Mikroorganismen-Populationen. Ein Vergleich zwischen verschiedenen Biotopen. Mar. Biol. 35:375-383.

13. Gocke, K. 1977. Comparison of methods for determining the turnover times of dissolved organic compounds. Mar. Biol. 42:131-141.

14. Hamilton, R. D., K. M. Morgan, and J. D. H. Strickland. 1966. The glucose uptake kinetics of some marine bacteria. Can. J. Microbiol. 12:995-1003.

15. Hamilton, R. D., and J. E. Preslan. 1970. Observations on heterotrophic activity in the Eastern Tropical Pacific. Limnol. Oceanogr. 15:395-401.

16. Hanson, R. B., and W. J. Wiebe. 1977. Heterotrophic activity associated with particulate size fractions in a *Spartina alterniflora* salt-marsh estuary, Sapelo Island, Georgia, USA, and the continental shelf waters. Mar. Biol. 42:321-330.

17. Harrison, M. J., R. T. Wright, and R. Y. Morita. 1971. Method for measuring mineralization in lake sediments. Appl. Microbiol. 21:698-702.

18. Herbland, A. 1975. Utilisation par la flore heterotrophe de la matiere organique naturelle dans l'eau de mer. J. Exp. Mar. Biol. Ecol. 19:19-31.

19. Hobbie, J. E., and C. C. Crawford. 1969. Respiration correction for bacterial uptake of dissolved organic compounds in natural waters. Limnol. Oceanogr. 14:528-532.

20. Hobbie, J. E., C. C. Crawford, and K. L. Webb. 1968. Amino acid flux in an estuary. Science 159:1463-1464.

21. Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

22. Hoppe, H. G. 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of microautoradiography. Mar. Biol. 36:291-302.

23. Iturriaga, V. R., and G. Rheinheimer. 1975. Eine einfache Methode zur Auszahlung von Bakterien mit aktivem Elektronentransportsystem in Wasser- und Sedimentproben. Kieler Meeresforsch. 31:83-86.

24. Novitsky, J. A., and R. Y. Morita. 1976. Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine vibrio. Appl. Environ. Microbiol. 32:617-622.

25. Novitsky, J. A., and R. Y. Morita. 1977. Survival of a psychrophilic marine vibrio under long term nutrient starvation. Appl. Environ. Microbiol. 33:635-641.

26. Seki, H. 1976. Method for estimating the decomposition of hexadecane in the marine environment. Appl. Environ. Microbiol. 31:439-441.

27. Stevenson, L. H. 1978. A case for bacterial dormancy in aquatic systems. Microbial Ecol. 4:127-133.

28. Vaccaro, R. F., and H. W. Jannasch. 1966. Studies on heterotrophic activity in seawater based on glucose assimilation. Limnol. Oceanogr. 11:596-607.

29. Vaccaro, R. F., and H. W. Jannasch. 1967. Variations in uptake kinetics for glucose by natural populations in seawater. Limnol. Oceanogr. 12:540-542.

30. Vaccaro, R. F. 1969. The response of natural microbial populations in seawater to organic enrichment. Limnol. Oceanogr. 14:726-735.

31. Wiebe, W. J., and D. F. Smith. 1977. Direct measurement of dissolved organic carbon release by phytoplankton and incorporation by microheterotrophs. Mar. Biol. 42:213-223.

32. Williams, P. J. LeB., and C. Askew. 1968. A method of measuring the mineralization by microorganisms of organic compounds in seawater. Deep-Sea Res. 15:365-375.

33. Williams, P. J. LeB., T. Berman, and O. Holm-Hansen. 1976. Amino acid uptake and release by marine heterotrophs. Mar. Biol. 35:41-47.

34. Williams, P. J. LeB., and R. W. Gray. 1970. Heterotrophic utilization of dissolved organic compounds in the sea. II. Observations on the responses of heterotrophic marine populations to abrupt increases in amino acid concentration. J. Mar. Biol. Ass. U.K. 50:871-88.

35. Wright, R. T. 1973. Some difficulties in using ^{14}C-organic solutes to measure heterotrophic bacterial activity, p. 199-220. *In* H. L. Stevenson and R. R. Colwell (eds.), Estuarine microbial ecology. University of South Carolina Press, Columbia.

36. Wright, R. T. 1974. Mineralization of organic solutes by heterotrophic bacteria, p. 546-565. *In* R. R. Colwell and R. Y. Morita (eds.), Effect of the ocean environment on microbial activities. University Park Press, Baltimore.

37. Wright, R. T., and B. K. Burnison. Heterotrophic activity measured with radiolabelled organic substrates. Proc. ASTM Symposium, Native Aquatic Bacteria; Enumeration, Activity and Ecology, June 25, 1977, Minneapolis (in press).

38. Wright, R. T. 1978. On the measurement and significance of specific activity in the heterotrophic bacteria of natural waters. Appl. Environ. Microbiol. 36:297-305.

39. Wright, R. T., and J. E. Hobbie. 1966. The use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology 47:447-464.

40. Wright, R. T., and N. M. Shah. 1975. The trophic role of glycolic acid in coastal seawater. I. Heterotrophic metabolism in seawater and bacterial cultures. Mar. Biol. 33:175-183.

41. Wright, R. T., and N. M. Shah. 1977. The trophic role of glycolic acid in coastal seawater. II. Seasonal changes in concentration and heterotrophic use in Ipswich Bay, Mass., USA. Mar. Biol. 43:257-263.

STRUCTURAL CHANGES CAUSED BY ENVIRONMENTAL POLLUTION OR EUTROPHICATION IN BACTERIAL COMMUNITIES IN SEA AND LAKE WATER

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ABSTRACT

The structure of bacterial communities in the sea and lakes is changed by environmental pollution or eutrophication. The effect of increased concentration of organic matter upon the structure of bacterial communities in natural water was observed using various selective media and a continuous culture system. A new method for the enumeration in natural water of extremely oligotrophic bacteria which cannot be counted using conventional solid media or diluted liquid media supplemented with organic nutrients is presented. It was found that extremely oligotrophic bacteria were predominant in oligotrophic lake water.

INTRODUCTION

The distribution and species composition of heterotrophic bacteria in the sea and lakes are much influenced by environmental pollution. When a concentration of organic matter in water is increased by pollution the bacteria usually found in the unpolluted pristine environment decrease in number or disappear, and inversely, the bacteria which "prefer" high nutrient media increase in number (1, 8, 13). However, there are few unequivocal evidences available to document the relationship between the structure of the bacterial community and concentration of organic matter in natural water.

In this paper, an attempt is made to clarify the effect of an increase in concentration of organic matter in water by pollution on the structure of bacterial communities in the sea and lakes by using various selective media and a continuous culture system.

MATERIALS AND METHODS

SAMPLING STATIONS AND MEDIA

Water samples were collected at stations 3, 4 and 5 in Osaka Bay (Fig. 1) which were at 6, 21 and 50 km, respectively, offshore from the mouth of the Yodo River. The water samples from stations 3, 4 and 5 contained 5.1, 2.3 and 1.5 mg, respectively, of dissolved organic carbon per liter. Station A was in a heavily polluted site with 4.0 mg of dissolved organic caron.

The Z1 medium used for this field survey contained 5 g of peptone (Daigo), 0.5 g of yeats extract (Difco) and 13 g of agar (Difco) in one liter of aged sea water, the latter collected from an unpolluted area. The pH value was adjusted to 7.7-7.8. The Z/20 medium contained peptone and yeast extract at the strength of 1/2 of Z1 medium.

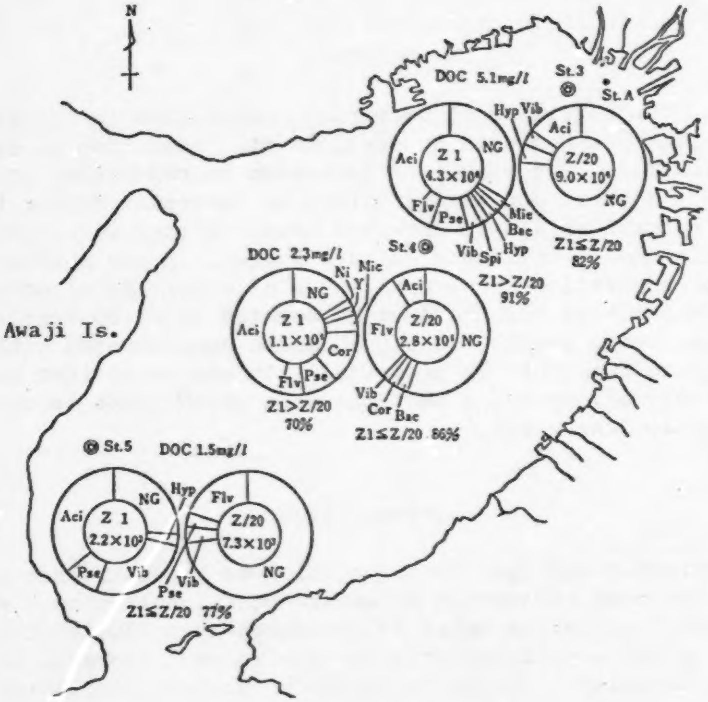

Figure 1. Location of sampling stations in Osaka Bay, bacterial number counted by using Z1 and Z/20 media and bacterial flora at each station. Concentration of organic matter expressed as DOC in surface water at each station is also shown.

CONTINUOUS CULTURE SYSTEM

The culture vessel was charged with 500 ml of raw sea water from station A and filtered through a stainless steel net (20 µm mesh) to remove detrital particles and zooplankters. The reservoir was filled with 5 liters of the filter-sterilized sea water collected at the same station. A second culture vessel was charged with 500 ml of unsterilized diluted sea water, which consisted of one part of the polluted sea water (DOC: 4.0 mg C/ml) and nine parts of clean sea water (DOC: 0.9 mg C/ml) which was filtered through a 20 µm mesh stainless steel net. The reservoir was filled with 5 liters of the diluted sea water after being sterilized through 0.22 µm filter. The dilution rate was adjusted to 0.123 ~ 0.130. The incubation temperature was adjusted to the in-situ water temperature (12°C).

COUNTING METHODS

Figure 2 illustrates the scheme for counting various groups of heterotrophic bacteria including extremely oligotrophic bacteria in water of Lake Biwa.

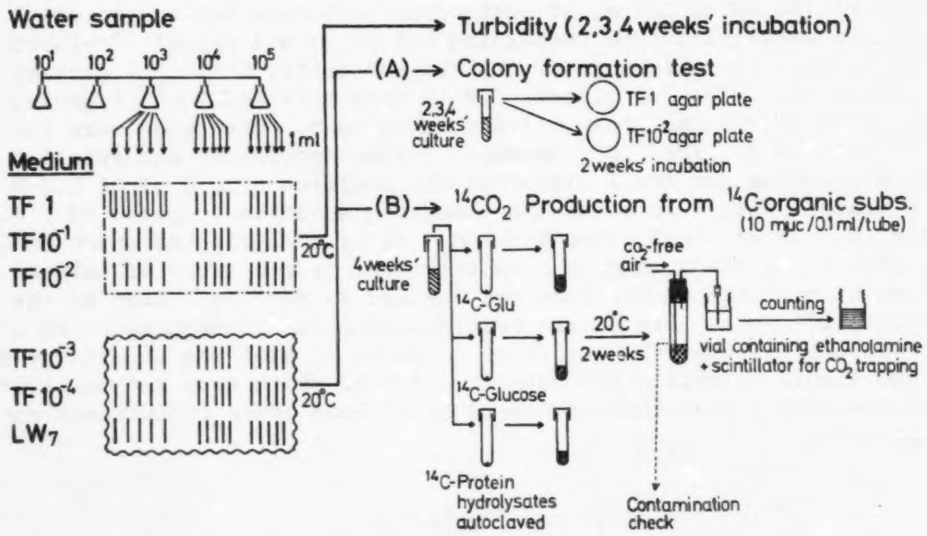

Figure 2. Diagrammatic scheme for enumeration of extremely oligotrophic bacteria.

After serial dilutions with sterilized artificial lake water (ALW: 0.5 mg NaCl, 0.38 mg KCl, 0.9 mg $CaCl_2 \cdot 4H_2O$, 0.1 mg $MgSO_4 \cdot 7H_2O$, 50 µg $MnCl_2 \cdot 4H_2O$, 40 µg $ZnCl_2 \cdot 7H_2O$, 1 µg $CuCl_2 \cdot 5H_2O$, 0.5 µg $CoCl_2 \cdot 6H_2O$, and 2 µg $FeCl_3$ per liter of distilled water), one ml of the diluted samples were inoculated into each of five test tubes containing TF1 medium (5 g trypticase (BBL) and 0.5 g yeast extract (Difco) in one liter of ALW at pH 7.0). The media $TF10^{-1}$, $TF10^{-2}$, TF10^{-3}, and TF10^{-4} were prepared to contain trypticase and yeast extract at the concentrations of 1/10, 1/100, 1/1,000 and 1/10,000, respectively, of those of the TF1 medium. Lake water from an oligotrophic area in Lake Biwa (Station 7) was filtered through a 0.22 μm filter, dispensed into test tubes, sterilized, and used as the lake water medium (LW_7 medium). The concentration of dissolved organic matter in the latter was 1.9 mg C/liter.

After incubating the inoculated media at 20°C for 14, 21 and 28 d, the growth of bacteria was detected by turbidity in TF1, TF10^{-1} and TF10^{-2} media. The most probable number of bacteria was calculated by use of Hoskins' table (3).

Since bacterial growth in TF10^{-3}, TF10^{-4} and LW_7 media could not be detected by turbidity, the following procedures were used.

a) Colony formation on agar plates: 0.1 ml of the culture at 14, 21 or 28 d incubation was spread onto each of duplicate agar plates of TF1 and TF10^{-2} media. The plates were incubated at 20°C for 14 d. The tube which gave more than 10 colonies on the plate was regarded as positive.

b) Detection of $^{14}CO_2$ production from organic compounds labelled with ^{14}C: After incubation for 28 d, 2.5 ml of culture in each tube was aseptically transferred to a sterilized tube containing 0.1 ml of 0.1 μCi/ml ^{14}C-labelled compound as a tracer level substrate. As the substrate, L-[U-^{14}C] glutamic acid (275 m Ci/m mol), [U-^{14}C] glucose (335 m Ci/m mol) and [U-^{14}C] protein hydrolysate (57 m Ci/m atom), respectively, were used. The tubes were incubated at 20°C for 14 d. The $^{14}CO_2$ produced during incubation was expelled by bubbling with CO_2-free air for 1 min after the addition of 0.1 ml of 1.2 N HCl. The CO_2 was trapped with 10.5 ml of the absorbing solution composed of 1 ml of ethanolamine, 1.5 ml of ethylcellusolve and 8 ml of scintillator which contained 4 g PPO, 0.2 g POPOP, and 60 g napththalene in one liter of toluene. Contamination of bacteria in the tube was checked by putting a drop of the culture onto TF10^{-1} agar plate before acidification and incubating it at 20°C for 14 d. Radioactivity of the absorbing solution in vial was counted by an Aloka LSC-502 liquid scintillation counter. A tube which gave radioactivity of at least 40% higher than that of the blank without added culture was regarded as positive.

RESULTS AND DISCUSSION

BACTERIAL COMMUNITY STRUCTURE (4)

Figure 3 shows the bacterial counts obtained by using Z1 and Z/20 media. Heterotrophic bacteria were most abundant in water at station 3 and most sparse in water at station 5. The ratio of the Z/20 bacteria counted on Z/20 medium to the Z1 bacteria counted on Z1 medium increased in inverse proportion to the DOC value of water. In general, the ratio was larger in summer than in winter. The data indicate that higher populations of bacteria can be obtained from polluted waters using media rich in organic nutrients rather than that poor in organic nutrients, and vice versa for unpolluted waters.

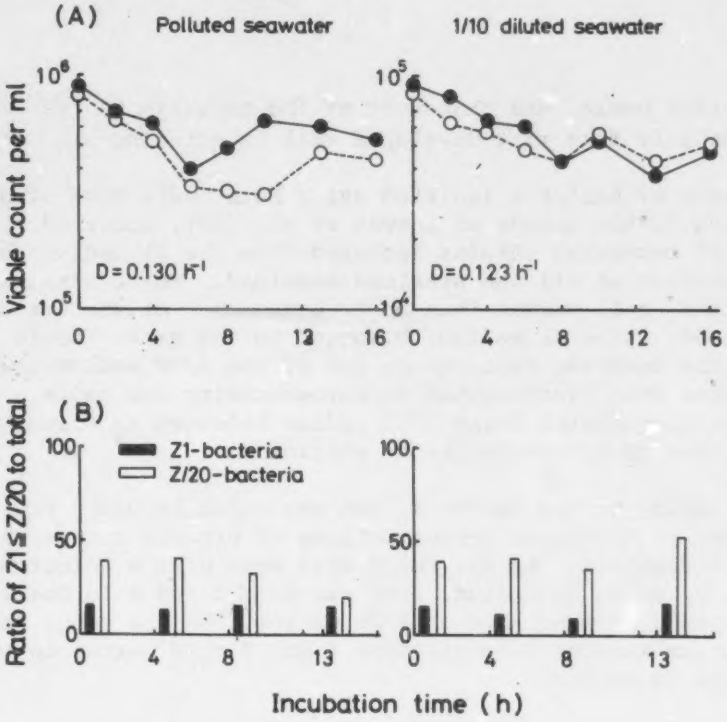

Figure 3. A. Viable counts of Z1 bacteria and Z/20 bacteria in the culture vessels which were fed with polluted sea water (DOC: 4.0 mg/l) and the 1/10 diluted sea water (DOC: 1.2 mg/l). ●= Z1 bacteria; ○= Z/20 bacteria.

B. Response of Z1 bacteria and Z/20 bacteria to different concentrations of organic nutrients. The Z1 ≤ Z/20 means the number of of bacteria which formed larger colonies on Z/20 plates than Z1 plates as second media.

Responses to different concentrations of organic nutrients of the bacteria from sea water of Osaka Bay were examined on Z1 and Z/20 media. Colonies formed on each of the original agar plates (Z1 and Z/20 media) were transferred to the second plates of Z1 and Z/20 media. After 1 or 2 wk incubation, it was possible to determine whether bacteria which formed colonies on the original plates grew well on transfers. Sizes of corresponding colonies of bacteria which could develop on both plates were compared. Bacteria which grew better on Z1 than Z/20 and those which showed superior growth on Z/20 than on Z1 was expressed as Z1>Z/20 and Z1≤Z/20, respectively. Of 49 strains isolated randomly from Z1 plates (Z1 bacteria), the ratio of Z1>Z/20 to the total was 91% in the water sample from station 3, and 70% from station 4. Of the 49 isolates from Z/20 plate (Z/20 bacteria), the ratio of Z1≤Z/20 was 82% at station 3, 86% at station 4 and 77% at station 5 (unpolluted area). Similar results were obtained in the other seasons. These data indicate that most of the bacteria enumerated on Z1 medium are ordinary heterotrophs that grow well on nutrient-rich media, and that most of the bacteria on Z/20 medium were relatively oligotrophic form that developed well on nutrient-poor media.

All the strains of bacteria isolated using both media were studied taxonomically according to the scheme of Shewan et al. (10), modified by Simidu (12). About 40% of bacterial strains isolated from the Z1 medium belonged to the genus *Acinetobacter* at all the stations examined. These strains of *Acinetobacter* grew better on Z1 medium than on Z/20 medium. At station 5, 28% of the strains isolated, using Z1 medium, belonged to the genus *Vibrio*. However, more than 50% of the bacteria isolated by use of the Z/20 medium grew neither on Z1 nor Z/20 media when transplanted to corresponding new media. Most of the remaining strains isolated using Z/20 medium belonged to *Flavobacterium* at stations 4 and 5, and to *Acinetobacter* at station 3.

Taxonomic grouping of the bacteria from sea water in Osaka Bay was made and their responses to different concentrations of organic nutrients on the second plates were examined. It was found that most of the *Acinetobacter* strains isolated, by using Z1 medium, from stations 3 and 4 in Osaka Bay grew better on the second Z1 medium than on Z/20 medium. On the other hand, most of the *Flavobacterium* strains from stations 4 and 5 grew better on the second Z/20 medium than on Z1 medium.

It has generally been recognized that high concentrations of peptone in agar media allow only a limited number of aquatic bacteria to form colonies (2, 11). Little work, however, has been done on physiological differences between bacteria developed on nutrient-poor media and those grown on nutrient-rich media. Our results indicate that bacteria which grow well on nutrient-poor media are different from bacteria showing better development on nutrient-rich media, not only in response to nutrient concentrations but also in the taxonomic types. It seems that bacteria from nutrient-poor media were temporarily suppressed by high concentrations of organic nutrients.

ANALYSIS OF STRUCTURAL CHANGES IN BACTERIAL COMMUNITY BY POLLUTION (5)

To obtain further information on the effect of organic substrates on the structure of bacterial communities in sea water we attempted an analysis with a continuous culture system charged with sea water containing organic substances in different concentrations.

During incubation, viable counting of Z1 and Z/20 bacteria in the culture vessels were made at intervals of 2 h; aliquots of the water samples were spread on Z1 and Z/20 media and incubated at 15°C. Colonies formed on each of the original agar plates were directly transplated to second Z1 and Z/20 plates. Changes in population densities of Z1 and Z/20 bacteria in the culture vessels during incubation are shown in Figure 3A. When polluted sea water was charged, the ratio in number of Z1 bacteria to Z/20 bacteria increased with incubation time, while in 1/10 diluted sea water the ratio did not change. Responses of the bacteria isolated from each of the plates to different concentrations or organic nutrients are shown in Figure 3B. The ratio of the Z1 bacteria which grew better on the second Z/20 medium than on the second Z1 medium to the total Z1 bacteria remained almost constant during 13 h incubation, regardless of the concentration of organic substances in the sea water. For Z/20 bacteria, however, the ratio of the bacteria which grew better on the second Z/20 media than on the second Z1 media to the total number decreased regularly during incubation when polluted sea water was charged, but increased gradually when the diluted sea water was charged.

The bacteria of the genus *Acinetobacter* (genus *Moraxella* was included), which predominated in polluted sea water, continued to be predominant in the culture vessel during 13 h incubation during charging with the polluted sea water. However, with 1/10 diluted sea water, containing organic nutrients in low concentration, density of *Acinetobacter* decreased during the first 8 h incubation (corresponding to the first retention time). In the latter instance, the genus *Vibrio* was predominant. These results may explain field observations wherein bacteria which grew better on nutrient-rich medium were predominant in the polluted sea water and the bacteria which grew well on nutrient-poor medium predominated in the unpolluted sea water.

GROWTH KINETICS OF BACTERIAL POPULATIONS (6)

Apparently two different bacterial populations (Z1 and Z/20 bacteria) compete for the organic substrates in sea water and the growth rates of the populations depend upon the concentration of organic substrates. Therefore, we examined the growth kinetics of Z1 and Z/20 bacteria with a continuous culture system. In this study, sea water samples containing organic substances in different concentrations (DOC: 1.0 mg/l, 1.1 mg/l, 1.8 mg/l and 5.0 mg/l) were fed to the culture vessels. The number of bacteria grown on Z1 and Z/20 media were plotted against the incubation time. According to the equation of Jannasch (9), actual growth rates were calculated from the washout rates $1/t \ln (x/x_0)$.

$$\mu = D + \frac{1}{t} \ln (x/x_0) \qquad [1]$$

where x_0 = population density at zero time (t=0), μ = specific growth rate (h^{-1}) and D = dilution rate (h^{-1}).

The relationship between the growth rates and the concentration of limiting substrate is illustrated in Figure 4. The competition between populations of Z1 and Z/20 bacteria was dependent on the concentration of organic substances as nutrients. The saturation curves of both populations crossed at approximately 5.0 mg C/l. When the concentration was less than 5.0 mg C/l, the population of Z/20 bacteria predominated. These growth rates were obtained with sea water samples collected in winter. In summer, the growth rates of Z1 bacterial populations were found to be 0.130 h^{-1} at 6.0 mg C/l (25°C) and 0.030 h^{-1} at 1.2 mg C/l (20°C), respectively, whereas the populations of Z/20 bacteria were 0.095 h^{-1} at 6.0 mg C/l and 0.090 h^{-1} at 1.2 mg C/l. These results suggest that the crossing point of the curves of Z1 bacteria and Z/20 bacteria shifts to lower concentration of organic substrates. Kinetics constants for the growth of both the populations (Z1 and Z/20 bacteria) were roughly estimated from the reciprocal plots for the populations of Z1 and Z/20 bacteria to be as follows, respectively: K_s^A = 3.0 mg C/l and μ_m^A = 0.19 h^{-1} (population of Z1 bacteria), and K_s^B = 1.2 mg C/l and μ_m^B = 0.15 h^{-1} (population of Z/20 bacteria). The data reveal that the larger μ_m was associated with larger K_s, and the smaller μ_m was associated with smaller K_s. Accordingly, the population of Z1 bacteria exhibited lower affinity toward organic substances at the low concentrations and the population of Z/20 bacteria showed higher affinity for organic substrates at the low concentrations. It is interesting that Jannasch arrived at the same conclusions by using mixed cultures of pure strains of aquatic bacteria (8).

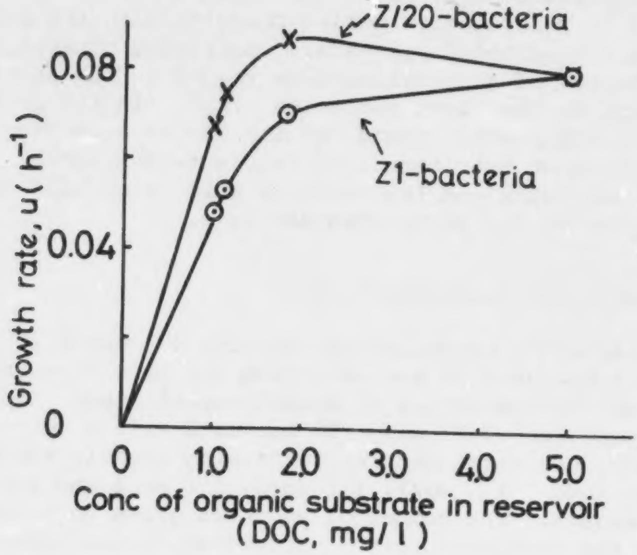

Figure 4. The growth rate-substrate concentration relationships of Z1 and Z/20 bacteria.

When the growth constants μ_m, K_s and Y (cell yield coefficient) were known, the steady state concentration (X) of bacterial population in the culture vessels could be predicted from the dilution rate (D) and the concentration of inflowing substrate (S_r), according to the following equation (2):

$$K_s \frac{D}{\mu_m - D} = S_r - \frac{X}{Y} \qquad [2]$$

When the population density of Z1 bacteria is X^A and that of Z/20 bacteria is X^B, the condition in which the Z1 bacteria become predominant in place of the Z/20 bacteria was

$$X^A > X^B \qquad [3]$$

By substituting [2] for [3]

$$D > \frac{\mu_m^B K_s^A - \mu_m^A K_s^B}{K_s^A - K_s^B} \qquad [4]$$

In this instance, the dilution rate to satisfy [3] is more than 0.11 per hour, when Y^A of the population of Z1 bacteria is equal to Y^B of the popula-of Z/20 bacteria. In the case of $Y^A > Y^B$, the dilution rate for satisfying [3] will be less than 0.11.

These experiments reveal that the structure of bacterial communities in the sea is controlled markedly by the concentrations of organic substances.

ENUMERATION OF EXTREMELY OLIGOTROPHIC BACTERIA IN LAKE WATER (7)

The Z/20 bacteria enumerated on an agar medium containing organic nutrients in relatively low concentrations in all likelihood play an important role in the biodegradation of organic matter in aquatic environments. However, there may exist in aquatic environments other groups of heterotrophic bacteria which cannot be counted by using diluted media such as Z/20 medium. Since the concentration of organic matter in the sea and lake waters (0.2 ~ 5 mg C/l) is usually much lower than that in Z/20 medium there may be some bacteria which can grow only in environments poor in organic nutrients. Such a group of organisms tentatively can be called "extremely oligotrophic" bacteria. Therefore, we attempted the detection and enumeration of such bacteria using water samples from various stations in oligotrphic and eutrophic areas of Lake Biwa.

In this study, many kinds of liquid media containing organic substrates in low concentrations were examined by use of extinction dilution methods. Criteria for the growth of bacteria included turbidity increase, colony formation on agar plates, and $^{14}CO_2$ production from glutamic acid, protein hydrolysate and glucose labelled with ^{14}C (Fig. 2). In water samples from station 0 in an eutrophic area of Lake Biwa (DOC: 4-5 mg C/l), the bacterial number counted by use of $TF10^{-1}$ medium was very large compared with that tabulated on other media. Numbers of extremely oligotrophic bacteria in this water sample were only 1.6% of the population densities obtained with $TF10^{-1}$. However, for water from station 7, in an oligotrophic area of Lake Biwa, the ratio of extremely oligotrophic bacteria to the $TF10^{-1}$ medium-counted bacteria was high (Table 1).

Of three kinds of organic substrates labelled with ^{14}C, glutamic acid and protein hydrolysate gave higher counts than glucose. This result might reflect the fact that the concentration of amino acids was higher (100 μg or more per liter as ninhydrin-positive substances) than that of glucose in the dissolved form (approximately 3 μg per liter) in the lake water; glucose might not be suitable substrate for the bacteria in the lake water. Conversely, the concentration of glucose used may have been insufficient for the extremely oligotraophic bacteria. To confirm this presumption, the $^{14}CO_2$ production activity of 12 strains of extremely oligotrophic bacteria from the $TF10^{-2}$, $TF10^{-3}$, $TF10^{-4}$ and LW_7 media cultures was examined by use of LW_7 medium, LW_7 medium with 1 ppm glucose and LW_7 medium with 10 ppm, to which 10 mμc of

TABLE 1. ENUMERATION OF HETEROTROPHIC BACTERIA IN WATER SAMPLES COLLECTED AT STATIONS 0 AND 7 IN LAKE BIWA

Medium	Criteria for growth	Bacterial number at			
		Station 0 (May 7, 1977)		Station 7 (June 9, 1977)	
		MPN (ml^{-1})	Ratio (%)*	MPN (ml^{-1})	Ratio (%)*
TF1	Turbidity	1.4×10^5	26	3.5×10^4	38
$TF10^{-1}$	Turbidity	5.4×10^5	100	9.2×10^4	100
$TF10^{-2}$	Turbidity	1.4×10^5	26	2.2×10^4	24
$TF10^{-3}$	(A) Colony formation on TF1 and $TF10^{-2}$	3.3×10^4	6.1	1.1×10^4	12
	(B) Respiration: glutamic acid	-	-	5.4×10^4	59
	protein hydrolysate	-	-	2.2×10^4	24
	glucose	-	-	2.4×10^2>	0.3>
$TF10^{-4}$	(A) Colony formation on TF1 and $TF10^{-2}$	4.5×10^3	0.9	4.9×10^3	5.3
	(B) Respiration: glutamic acid	1.3×10^4	2.4	3.5×10^4	38
	protein hydrolysate	1.3×10^4	2.4	1.4×10^4	15
	glucose	4.5×10^3	0.9	2.4×10^2>	0.3>

*Ratio of each count to $TF10^{-1}$ count.

[U-^{14}C]-glucose was added. It was found by this experiment that $^{14}CO_2$ was not produced from all the media employed. These results suggest that, compared with amino acids, glucose is not a suitable substrate for respiration of extremely oligotrophic bacteria in lake water. There is, however, a possibility that the TF media were deficient in some components required for growth of extremely oligotrophic bacteria. Therefore, the LW_7 medium prepared from filtered and sterilized lake water was used for enumeration of extremely oligotrophic bacteria (Table 2). The number of heterotrophic bacteria (B) and that of extremely oligotrophic bacteria (B - A) with LW_7 medium with ^{14}C-glutamic acid were 6.7 and 5.8 times, respectively, higher than that of ordinary heterotrophic bacteria counted by use of $TF10^{-1}$ medium. The concentration of dissolved organic matter in LW_7 medium was 1.9 mg C/l which was comparable to that of $TF10^{-3}$ medium.

TABLE 2. ENUMERATION OF HETEROTROPHIC BACTERIA IN WATER SAMPLES FROM STATION 7 IN LAKE BIWA (JULY 7, 1977)

Medium	Criteria for growth	Bacterial number	
		MPN (ml^{-1})	Ratio (%)*
TF1	Turbidity	1.7×10^4	71
$TF10^{-1}$	Turbidity	2.4×10^4	100
$TF10^{-2}$	Turbidity	4.9×10^3	20
$TF10^{-3}$	(A) Colony formation on TF1 and $TF10^{-2}$	1.7×10^3	7.1
	(B) Respiration: glutamic acid	1.7×10^3	71
	protein hydrolysate	1.7×10^4	
	glucose	1.3×10^3	
$TF10^{-4}$	(A) Colony formation on TF1 and $TF10^{-2}$	5.5×10^2	2.3
	(B) Respiration: glutamic acid	4.0×10^3	17
	protein hydrolysate	2.4×10^3	
	glucose	$2.4 \times 10^2>$	
LW_7	(A) Colony formation on TF1 and $TF10^{-2}$	1.7×10^4	46
	(B) Respiration: glutamic acid	1.6×10^5	667
	protein hydrolysate	9.2×10^4	
	glucose	1.3×10^4	

*Ratio of each count to $TF10^{-1}$ count.

Strains of extremely oligotrophic bacteria from oligotrophic waters in Lake Biwa showed a relatively high growth rate (generation time, approximately 6 h) and a very low maximum cell yield (less than 10^6 cells/ml) in a liquid medium containing organic matter in low concentration (approximately 2 mg C/l). All bacteria grown in $TF10^{-1}$ medium were able to grow in LW_7 medium, such organisms may be called facultatively oligotrophic bacteria.

Data reported here suggest that some growth-promoting factors were contained in natural lake water and that extremely oligotrophic bacteria were predominant in water in oligotrophic areas of Lake Biwa and probably contribute to the biodegradation processes in the lake.

LITERATURE CITED

1. Akagi, Y., N. Taga, and U. Simidu. 1977. Isolation and distribution of oligotrophic marine bacteria. Can. J. Microbiol. 23:981-987.

2. Fònden, R. A. 1968. Yeast-extract peptone agar for the determination of heterotrophic bacteria in lakes. Vatten 2:161-166.

3. Hoskins, J. K. 1934. Most probable numbers for evaluation of coli-aerogenes tests by fermentation tube method. U.S. Public Health (Service) Repts. 49:393-405.

4. Ishida, Y., and H. Kadota. 1974. Ecological studies on bacteria in the sea and lake waters polluted with organic substances. I. Responses of bacteria to different concentrations of organic substances. Bull. Japan. Soc. Sci. Fish. 40:999-1005.

5. Ishida, Y., and H. Kadota. 1975. Ecological studies on bacteria in the sea and lake water polluted with organic substances. II. Analysis of bacterial flora by use of a chemostat. Bull. Japan. Soc. Sci. Fish. 41:957-960.

6. Ishida, Y., and H. Kadota. 1975. Ecological studies on bacteria in the sea and lake waters polluted with organic substances. III. Growth kinetics of the bacteria populations showing different responses to dissolved organic substances. Bull. Japan. Soc. Sci. Fish. 41:961-964.

7. Ishida, Y., and H. Kadota. 1978. A new method for enumeration of oligotrophic bacteria in lake water. Arch. Hydrobiol. (in press).

8. Jannasch, H. W. 1967. Enrichments of aquatic bacteria in continuous culture. Arch. Mikrobiol. 59:165-173.

9. Jannasch, H. W. 1969. Estimations of bacteria growth rates in natural waters. J. Bacteriol. 99:156-160.

10. Shewan, J. M., G. Hobbs, and W. Hodgkiss. 1960. A determinative scheme for the identification of certain genera of gram-negative bacteria, with special reference to the Pseudomonadaceae. J. Appl. Bacteriol. 23:379-390.

11. Sieburth, J. McN. 1967. Seasonal selection of estuarine bacteria by water temperature. J. Exp. Mar. Biol. 1:98-121.

12. Simidu, U. 1974. Taxonomy of marine bacteria, p. 45-65. *In* N. Taga (ed.), Kaiyo biseibutsu (marine microbes). Univ. Tokyo Press, Tokyo.

13. Simidu, U. 1975. In situ analysis of microbial flora changes in polluted sea, p. 50-60. *In* H. Kadota, N. Taga, and M. Sakai (eds.), Ecosystem in the sea and microorganisms. Koseisha-Koseikaku, Tokyo.

NITROGEN/CARBON RATIOS AND RATES OF AMMONIA TURNOVER IN ANOXIC SEDIMENTS

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ABSTRACT

The net rate of ammonia production (d-i) was measured in sediment sections, at in-situ temperatures. Addition of ^{15}N-labeled ammonia made possible the measurement of total ammonia turnover (d) and of ammonia incorporation into cells (i). The molar N/C ratio (Ns) in detrital material undergoing breakdown, and the rate of carbon oxidation (Co) or sulfate reduction (Sr) were calculated, using known values for the N/C ratio in cells (Nc) and the efficiency of carbon incorporation into cells (E), from the following: $Ns = E \times Nc \times d/i$

$$2\ Sr = Co = i(1-E)/(E \times Nc)$$
$$2\ Sr = Co = (d-i)(1-E)/(Ns-Nc \times E)$$

The calculated Ns values did not always correspond to the actual N/C ratio of the organic detritus in the sediment. The data are consistent with the hypothesis that surface detritus of an initially high N/C ratio progressively loses nitrogen as it is mixed downwards and finally results in a net uptake of ammonia in the lower sediment strata.

INTRODUCTION

Detritus, containing carbon and nitrogen, is degraded and used by bacteria, partially as a source of energy and partially for incorporation into new cellular material. The net production (or consumption) of degraded nitrogen compounds depends on the N/C ratio of the detritus and of the cells, and on the efficiency with which detrital material is assimilated into cellular compounds.

These relationships are examined and are used to illustrate how rates of ammonia turnover, incorporation and net production may be used to calculate N/C ratios in the substrate and rates of carbon oxidation (or sulfate reduction) in anoxic marine sediments.

METHODS

The rates of net ammonia production (d-i) in sediment sections were measured at in-situ temperatures, where diffusional losses could not occur. Addition of ^{15}N-labeled ammonia to these samples made possible the measurement of total ammonia production (d), using net ammonia production rate to determine the dilution of label by the changing pool. The difference between the two rates gave the rate of ammonia incorporation into bacterial cells (i) at any depth (Blackburn, unpublished results). Organic carbon in sediments was determined by combustion in a Leco furnace, correcting for the contribution from carbonates. Total nitrogen was determined, following Kjeldahl digestion (3). Organic nitrogen was obtained by correcting for extractable and non-extractable ammonia. The sediments were sampled from the stations described by Jørgensen (5).

RESULTS AND DISCUSSION

The fate of C and N, following the breakdown of detritus by hydrolytic, fermentative and sulfate-reducing bacteria is shown in Figure 1. Carbon is metabolized at a rate Cd, oxidized at a rate Co and incorporated into cells at a rate Ci (Cd = Co + Ci; Ci/Cd = E, the efficiency of incorporation). Similarly, nitrogen is liberated at a rate d (d/Cd = Ns, the N/C ratio of the substrate), assimilated at a rate i (i/Ci = Nc, the N/C ratio of bacterial cells) and excess ammonia is made available at a rate d-i (net production rate). It is assumed that bacteria assimilate nitrogen mostly in the form of ammonia, thus allowing the following relationships to be established:

1) Ns = Nc x E x d/i
2) Co = i(1-E)/(Nc x E)
3) Co = (d-i)(1-E)/(Ns-Nc x E)
4) Co = 2Sr

where Sr = the rate of sulfate reduction by carbon at the oxidation-reduction level of CH_2O.

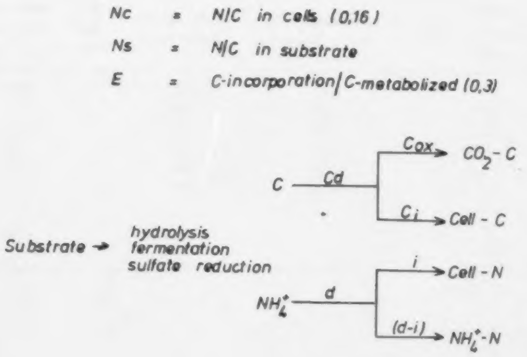

Figure 1. The processes involving the production of cells, carbon dioxide and ammonia from detritus in sediment.

In order to calculate Ns and Co from equations 1 and 2 it is necessary to know the values for Nc and E, in addition to the ammonia rates. The value for Nc = 0.16 (Fig. 1) is consistent with quoted values for bacterial cells (1, 2). The value E = 0.31 is based on the calculations in Table 1. The assignment of approximately 1 ATP to the oxidation of acetate by sulfate fits with the efficiency of acetate assimilation by *Desulfotomaculum acetoxidans* (6).

TABLE 1. EFFICIENCY OF CARBON INCORPORATION ASSOCIATED WITH SULFATE REDUCTION[a]

Process	G_0' kcal mol^{-1}	ATP available[b] for synthesis	C atoms dissimilated	C atoms assimilated
Fermentation:				
100 hexose C → lactate + cells	-52	2	88	12
SO_4^{2-} reduction:				
88 lactate → CO_2 + cells	-38	2	69	19
Total:				
100 hexose C → CO_2 + cells			69	31

[a] It is assumed that 1 mol ATP will form 10 g cells or 0.42 mol of organic C.

[b] Per mol substrate dissimilated.

The data in Table 2 illustrate how d, d-i and i vary with depth in an average of four sediments from the Limfjord in August 1976, and how these values may be used to calculate the N/C ratio in the substrate and the rates of carbon oxidation or sulfate reduction, using the equations derived in equations 1 through 4.

TABLE 2. RATES OF AMMONIA TURNOVER

Depth (cm)	NH_4^+ increase[a] (d-i)	NH_4^+ production[a] (d)	NH_4^+ incorporation[a] (i)	N/C molar (N_s) calculated	N/C molar measured	SO_4^{2-} reduction (Sr) calculated from eq. 2 and 4	SO_4^{2-} reduction[a] (Sr) calculated from eq. 3 and 4
0-2	74	131	57	.11	.11	396	423
2-4	49	95	46	.10	.09	320	418
4-6	40	78	38	.10	.10	264	274
6-8	37	103	66	.08	.10	459	253
8-10	15	44	29	.08	.09	201	128
10-12	4	49	44	.06	.09	306	34
Total for 12 cm	438	1000	560			3992	3060

[a] Rates are in n mol cm^{-3} day^{-1}.

[b] Using N_s = measured N/C molar ratio in sediment.

There was a decrease in total ammonia production, with depth of sediment, but there was still considerable activity at 10 to 12 cm. At this depth, however, there was little net ammonia production as ammonia incorporation was quite high. The latter rate showed surprisingly little variation with depth down to 12 cm. The calculated Ns decreased with depth and was generally lower than the measured value of sediment N/C molar ratio.

The calculated rate of sulfate reduction (Sr) from equations 2 and 4 showed little decrease with depth, since it was determined from the rate of ammonia incorporation. The values of Sr, calculated from equations 3 and 4, decreased with depth in a manner consistent with the values measured in the same sediments (5). The respective values of 39.9 and 30.6 mmol $m^{-2}day^{-1}$, down to 12 cm, are somewhat higher than the measured yearly average of 9.5 mmol $m^{-2}day^{-1}$ (5). The utilization of equation 3 has considerable advantages as ^{15}N tracer experiments are not necessary for the measurement of d-i, which may even be derived from the change in flux in ammonia, between two adjacent strata. It may thus be calculated from an ammonia concentration profile, provided that it is an undisturbed sediment and that the diffusion coefficient is known. The value of Ns must be known to use equation 3. The data in Table 2 demonstrate that the actual Ns value is not the same as the N/C ratio of the sediment, and can be significantly different (Fig. 2). It seems likely that, in sediments where vertical mixing does not occur, the Ns value must equal the sediment N/C ratio.

The data in Figure 2 illustrate how the net rate of ammonia production and the calculated N/C ratio of substrate (Ns) at various depths may be used to explain some aspects of ammonia concentration profiles at station 5 in the Limfjord.

The concave interstitial ammonia concentration profile at 6-8 cm for May was associated with a negative net ammonia production at 4-8 cm and a low N/C in the substrate from 4-12 cm. This contraction in ammonia concentrations continued into June, even though there was considerable net ammonia production in the top 4 cm of sediment, associated with a high Ns in this region, presumably due to the deposition of fresh algal cells on the surface. During July and August the ammonia concentrations increased (unpublished results) but by September they had contracted again, due partially to the negative net ammonia production, associated with the low Ns of the substrate undergoing decomposition from 2-12 cm.

These patterns fit with the hypothesis that algal cells of a high N/C content were decomposed at the sediment surface, with preferential decomposition of protein and nucleic acids, liberating ammonia. The partially decomposed cells, rich in structural materials of low N/C ratio, gradually mixed downwards and were degraded in preference to the old and recalcitrant organic material in the sediment, which had a higher N/C ratio (Fig. 2, top right). When the Ns was less than 0.05 (when $i > d$, $E = 0.03$ and $Nc = 0.16$) there would be net ammonia uptake.

The hypothesis depends on the mixing of surface detritus downwards to at least 12 cm. This is consistent with the observation that sulfate reduction in these sediments was higher than could be explained by the oxidation of detritus to which no new additions were made (5). The role of macrofauna in sediment irrigation and mixing has recently been demonstrated (4) Additional evidence for sediment mixing is derived from the fact that the measured values

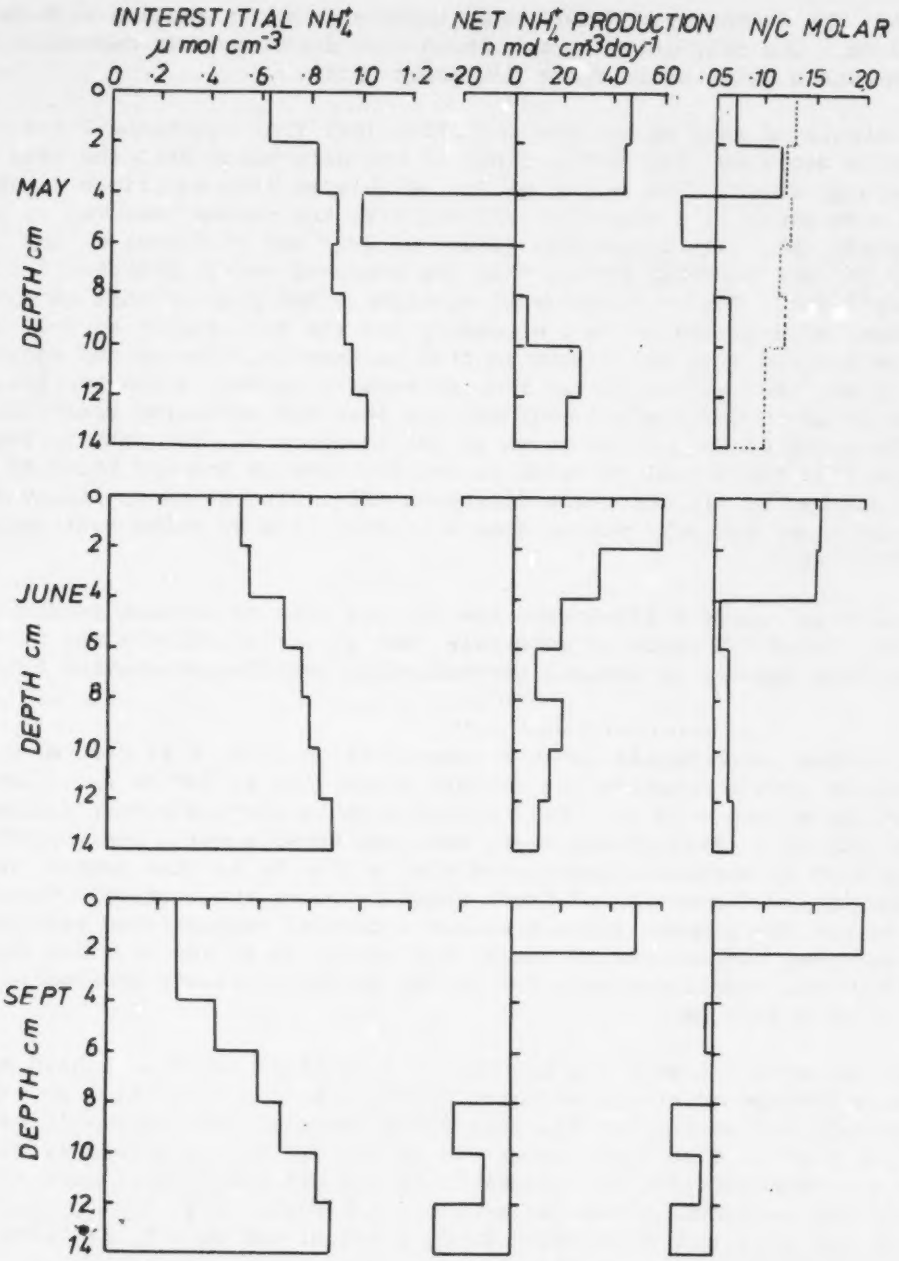

Figure 2. Interstitial ammonia concentrations, net ammonia production rates and Ns (N/C molar ratios) at 2 cm intervals in the Limfjord station 5, for May (12°C), June (16°C) and September (12°C) in 1977. The broken line in the May values of Ns (top right) shows the sediment N/C molar ratio.

for the rates of net ammonia production, particularly in the summer months, are not consistent with the observed changes in the ammonia concentrations. The ammonia concentration gradients are not sufficient to account for the disappearance of ammonia from the lower strata, unless very high diffusion coefficients are postulated for ammonia.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Bardowskiy, O. K. 1965. Sources of organic matter in marine basins. Mar. Geol. 3:5-31.

2. Bardowskiy, O. K. 1965. Accumulation of organic matter in bottom sediments. Mar. Geol. 3:33-82.

3. Bremner, J. M. 1960. Determination of nitrogen in soil by the Kjeldahl method. J. Agr. Sci. 55:1-23.

4. Goldhaber, M. B., R. C. Aller, J. K. Cochran, J. K. Rosenleld, C. S. Martens, and R. A. Berner. 1977. Sulfate reduction, diffusion, and bioturbation in Long Island Sound sediments: report of the FOAM group. Am. J. Sci. 277:193-237.

5. Jørgensen, B. B. 1977. The sulfur cycle in a coastal marine sediment (Limfjorden, Denmark). Limnol. Oceanog. 22:814-832.

6. Widdel, F., and N. Pfennig. 1977. A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, *Desulfotomaculum* (emend.) *acetoxidans*. Arch. Microbiol. 112:119-122.

EFFECT OF CHEMICAL STRUCTURE AND CONCENTRATION ON MICROBIAL DEGRADATION IN MODEL ECOSYSTEMS

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ABSTRACT

Increasing the number of chlorines on benzoates increases their resistance to attack by microorganisms in sewage. Populations of bacteria able to grow on readily biodegradable benzoates multiply as the chemicals disappear from model sewage ecosystems. The major bacteria in sewage growing on unsubstituted benzoate will not grow on simple chlorinated benzoates. The addition of benzoate to sewage does not lead to the loss of 2,4-di- and 2,3,6-trichlorobenzoates. During the decomposition of *m*-chlorobenzoate, 4-chlorocatechol and 5-chlorosalicylate appear. In stream water, parts-per-trillion concentrations of *p*-chlorobenzoate, dimethylamine, and diethanolamine are quickly converted to carbon dioxide. In contrast, parts-per-million or low parts-per-billion concentrations of the herbicide 2,4-D are converted to carbon dioxide very slowly, although higher concentrations are destroyed readily. The data suggest that chemicals may be attacked only slowly if present in waters at very low concentrations.

Corresponding to the continuing rise in production and use of synthetic organic chemicals is an increased discharge of these chemicals into natural waters, either deliberately or inadvertently, as domestic and industrial wastes, runoff from agricultural operations, or accidental spillage. Most of these compounds are probably rendered harmless soon after their introduction into aquatic environments. A surprisingly large number of organic chemicals, however, may not be metabolized; among those that are transformed microbiologically, the products may be more toxic than the original compound, or they may persist for longer periods of time. Although a considerable amount of information has accumulated about the metabolism of these organic compounds by pure or mixed cultures, little is known about their fate in natural ecosystems, both aquatic and terrestrial.

Several factors govern the susceptibility of organic chemicals to microbial destruction in nature. In the present investigation, interest is directed to the effects of chemical structure and concentration on the biodegradation of organic compounds. Ample evidence exists of a relationship between the structure of an organic chemical and its susceptibility to microbial attack (1, 11). Moreover, Jannasch (8) and Shehata and Marr (12) demonstrated that stable, axenic microbial populations could not be maintained in continuous culture at low concentrations of glucose and other simple organic molecules. However, no systematic investigations have been conducted of the influence of low substrate concentration on the biodegradation of pollutants in artificial microcosms or ecosystems.

The present study was undertaken, therefore, to assess the effects of chemical structure and concentration on the biodegradation of organic molecules in artificial aquatic microcosms. The experimental systems used were designed to reflect the complex interactions in natural microbial communities.

MATERIALS AND METHODS

Surface water samples were taken from Fall Creek, a tributary of Cayuga Lake in central New York. Sewage samples were taken from the primary settling tank of the water treatment plant at Ithaca, New York. All samples were processed within 2-4 h after collection.

An activated-sludge die-away system was used in studies of the degradation of benzoate and chloro-substituted benzoates. Primary influents were incubated at room temperature in 1.0 liter quantities in 2-liter Erlenmeyer flasks, and the suspensions were aerated with air that had been passed through a saturated solution of $KMnO_4$. Sewage was amended with the test chemical to a final concentration of 100 mg/liter. Cell counts were performed by a most-probable-number method for enumerating populations of aromatic degraders (7).

Benzoate and chlorinated benzoate were determined by gas-liquid chromatography using a Perkin-Elmer gas chromatograph, model 3920B, fitted with a flame-ionization detector. Samples were saturated with NaCl, acidified with HCl, and extracted with anhydrous ether. The ether extracts were concentrated to a volume of 2-5 ml. Benzoate and monochlorinated benzoates were analyzed on a teflon-lined stainless steel column (0.6 m x 2mm, i.d.) packed with 10% DEGA on 80/100 mesh Chromosorb G (H.P.) at a column oven temperature of 190°C. Dichlorinated and trichlorinated benzoates were derivatized with bis(trimethylsilyl) trifluoroacetamide (Pierce Chemical Co.) in demethylformamide after concentration of the ether extracts, and the derivatives were analyzed on a 1.8 m x 2 mm i.d. teflon-lined stainless steel column packed with 3% OV17 on 80/100 mesh Chromosorb G at a column oven temperature of 130°C. Metabolic intermediates were identified using a Finnigan 3300 Quadrupole mass spectrometer (electron impact 70 eV) after derivatization with diazomethane. Spectrophotometric determinations were performed by the method of Alexander and Lustigman (2), and dihydroxy compounds were measured by the method of Arnow (4).

The degradation of low concentrations of organic compounds was studied using a stream die-away system. Stream water was collected aseptically and analyzed for pH and total alkalinity by standard methods (3). The water was amended with an inorganic nutrient supplement (13) modified as follows: $NaNO_3$ and $NaHCO_3$ were omitted, Zn was added at the same molarity as $ZnSO_4 \cdot H_2O$ rather than $ZnCl_2$, and NH_4Cl was added at 8.0 mg/liter.

Microbial activity was assayed by measurement of $^{14}CO_2$ produced from ^{14}C-labeled substrates. Stream water was incubated at 28.5°C in 100- or 200-ml volumes in 500-ml filtering flasks modified to permit connection to a gas train subsequent to incubation. The flasks were incubated in the dark to prevent photodecomposition of added organic chemicals and fixation of $^{14}CO_2$ by algae. Although the flasks were not shaken, measurements of dissolved oxygen (3) indicated that the stream water remained aerobic.

Individual organic compounds were added to the flasks at various concentrations. After specified periods of time, the contents of the flasks were acidified with 0.12 N H_2SO_4, the flasks were connected to a source of N_2, and the CO_2 in the flasks was flushed out and trapped in ethanolamine. If necessary, $NaHCO_3$ was added immediately before acidification to raise the concentration of bicarbonate plus carbonate to at least 1.0 mM. Control experiments indicated that the recovery of $^{14}CO_2$ was quantitative, and that no degradation of any compound to $^{14}CO_2$ occurred in autoclaved stream water. None of the ^{14}C-labeled compounds tested nor any impurities in their formulation were sufficiently volatile to be flushed out with N_2 after acidification and trapped in ethanolamine. This method for assessing biodegradation of organic chemicals does not distinguish between degradation to $^{14}CO_2$ and possible conversion to other volatile metabolites that may be trapped in ethanolamine.

Samples (1.0 g) of ethanolamine containing trapped $^{14}CO_2$ were added to 9 ml of scintillation fluid (6), and the radioactivity was measured in a Beckman LS-100C liquid scintillation spectrometer. The data were corrected for quench by the external standard/channels ratio method and expressed as dpm. The counting efficiency of ethanolamine (1.0 g) in Bray's scintillation fluid was about 75%.

The three monochlorinated benzoic acid isomers, 3,4- and 2,4-dichlorobenzoic acids, and 2,4-dichlorophenoxyacetic acid were obtained from Aldrich Chemical Co. Scintillation grade ethanolamine and unlabeled dimethylamine hydrochloride were obtained from Eastman Organic Chemicals, diethanolamine from Fluka AG, and sodium benzoate from Merck. 2,3,6-Trichlorobenzoic acid (Pfaltz and Bauer) was recrystallized from hot water.

The sources and specific activity of the radiochemicals used in this study were as follows: dimethylamine-^{14}C hydrochloride (Amersham), 54 mCi/mmol; diethanolamine- ^{14}C (New England Nuclear), 20.8 mCi/mmol; *p*-chlorobenzoic acid-ring ^{24}C (California Bionuclear), 10.5 mCi/mmol; 2,4-dichlorophenoxyacetic acid-acetate 2-^{14}C (Amersham), 32 mCi/mmol.

RESULTS

The primary degradation of the chloro-substituted benzoates in sewage was investigated initially. The results are presented in Figure 1. Primary degradation was more rapid with benzoic acid than with the chlorinated analogues. *o*-Chlorobenzoate was metabolized more rapidly and disappeared sooner than did *p*- and *m*-chlorobenzoate. Of the polychlorinated compounds tested, 3,4-dichlorobenzoate disappeared in 9 days, but no loss of 2,4-dichlorobenzoate or 2,3,6-trichlorobenzoate was detected, even after an additional 18 days.

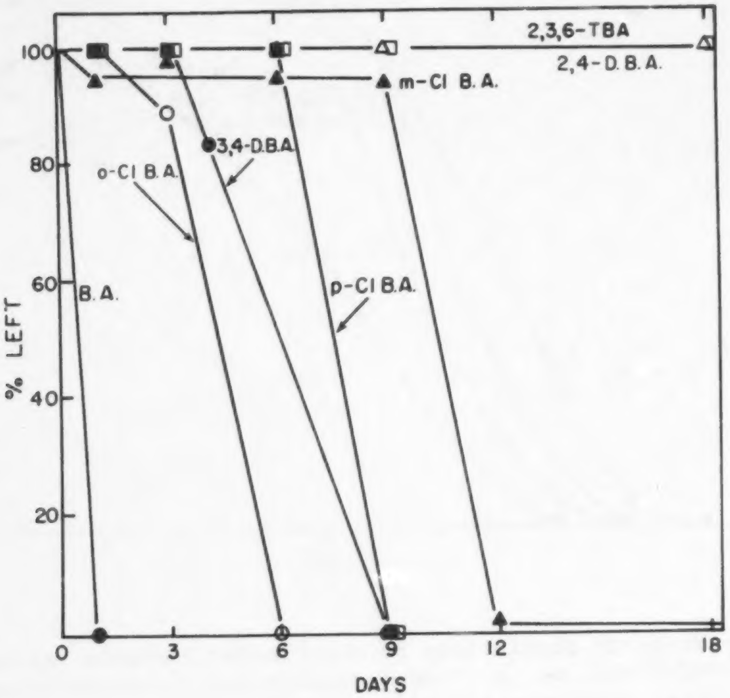

Figure 1. Degradation of benzoate and *m*-, *p*-, *o*-, 2,4-di-, 3,4-di-, and 2,3,6-trichlorobenzoates in sewage. Sewage was amended to an initial concentration of 100 μg/ml of each test compound. Abbreviations: B.A., benzoate; *o*-, *p*-, and *m*-Cl B.A. are the *o*-, *p*-, and *m*-chlorobenzoates; 3,4- and 2,4-D.B.A. are 3,4- and 2,4-dichlorobenzoates; and 2,3,6-TBA is 2,3,6-trichlorobenzoate.

To determine whether disappearance of the compound corresponded to an increase in a population capable of utilizing aromatic compounds, sewage was amended with 100 μg/ml of the chlorinated compounds, and cell numbers were determined. The results in Figure 2 demonstrate that the numbers of bacteria capable of using the chlorinated compounds as carbon and energy sources for growth rose in sewage amended with the benzoates previously shown to disappear. The populations of chlorinated benzoate utilizers were initially less than 10^2 cells/ml but increased to a maximum of about 10^6 cells/ml. In contrast, there was no increase in populations capable of metabolizing 2,4-di- and 2,3,6-trichlorobenzoates.

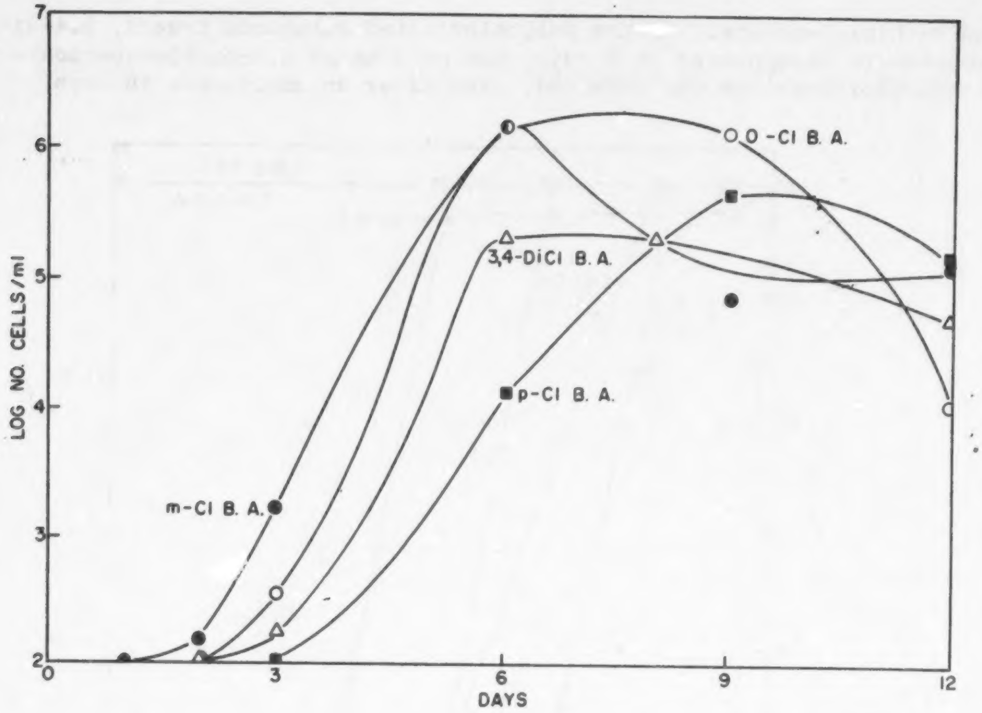

Figure 2. Increase in populations of chlorinated benzoate utilizers in sewage amended with *o*-, *m*-, and *p*-chloro- and 3,4-dichlorobenzoates to an initial concentration of 100 µg/ml.

To determine what effect benzoate metabolism might have on the proliferation of the chlorobenzoate utilizers, sewage was amended with 100 µg of sodium benzoate/ml and monitored for changes in populations of both benzoate and chlorobenzoate utilizers. The results in Figure 3 show that the number of benzoate utilizers increased rapidly in the first 24 h but then declined somewhat. In contrast, the size of the chlorobenzoate-utilizing populations was unaffected by the amendment and remained at less than 10^2 cells/ml.

To assess whether the more refractory chlorinated compounds are modified in the presence of a utilizable and structurally related substrate, two flasks containing sewage were amended with sodium benzoate (100 µg/ml) and either 2,4-di- or 2,3,6-trichlorobenzoate. The number of cells and the percent of the test compound remaining as a function of time are shown in Figure 4. The results show that there was no disappearance of either 2,4-dichlorobenzoate or 2,3,6-trichlorobenzoate as the benzoate was being destroyed. No increase in the population capable of growing at the expense of the chloro-substituted benzoates was evident, whereas, concomitant with benzoate degradation was an increase in the population capable of using this compound for growth.

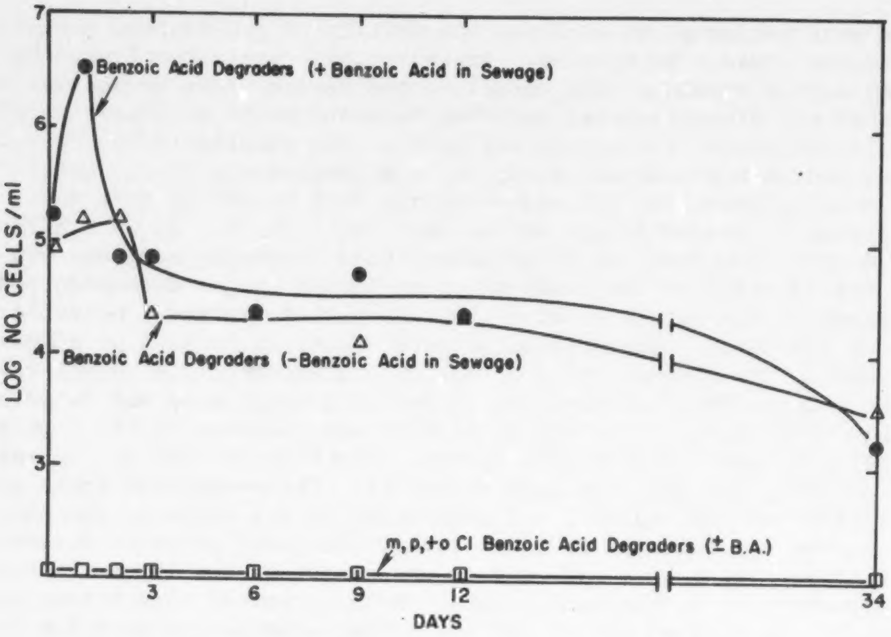

Figure 3. Populations of benzoate and chlorobenzoate utilizers in sewage amended with 100 μg benzoate/ml or without benzoate addition.

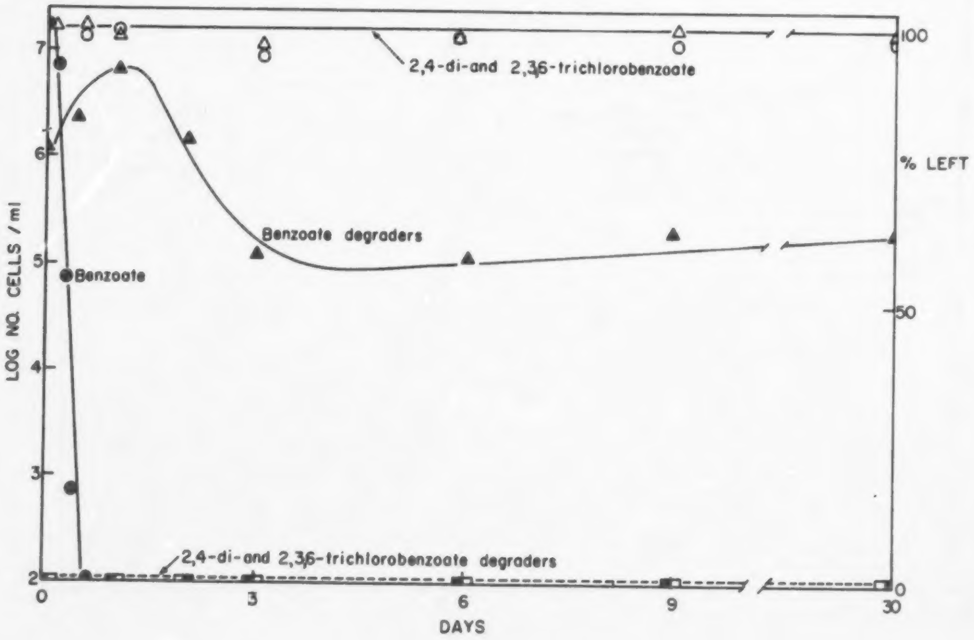

Figure 4. Effect of benzoate on the degradation of 2,4-di- and 2,3,6-trichlorobenzoates in sewage.

Studies were performed to evaluate the ability of pre-adapted populations to respond to new organic substrates. Pre-adaptation was accomplished by initially adding sodium benzoate (100 μg/ml) to the sewage. The sewage was monitored for substrate disappearance, and when benzoate could no longer be detected, *m*-chlorobenzoate (100 μg/ml) was added. The results in Figure 5 demonstrate that sodium benzoate was degraded in approximately 12 h. After the addition of *m*-chlorobenzoate, its concentration fell by 18% in four days, but no further change in concentration was evident until day 8. At 24 h after the addition of *m*-chlorobenzoate, an *ortho*-substituted dihydroxy compound was detected, and the identity of the product was confirmed by gas chromatography-mass spectrometry. The methyl ester of this metabolite showed a molecular ion with an m/e of 172 and a fragmentation pattern identical to that of authentic 4-chlorocatechol. The concentration of the chlorocatechol (9.0 μg/ml) remained constant until 48 h, after which it declined until none was detectable at 4 d. During this time, there was no significant increase in the population capable of using *m*-chlorobenzoate for growth. After day 8, the chlorobenzoate disappeared quickly, and none was left at day 12. The sewage was again amended with *m*-chlorobenzoate (100 μg/ml), and monitoring of the compound showed that the chlorobenzoate was degraded in 3 d. During the rapid phase of decomposition of *m*-chlorobenzoate, a second product was detected, and it was identified by mass spectrometry as 5-chlorosalicylate. Methylation of this metabolite gave a molecular ion with an m/e of 186 and a fragmentation pattern identical to that of authentic 5-chlorosalicylate.

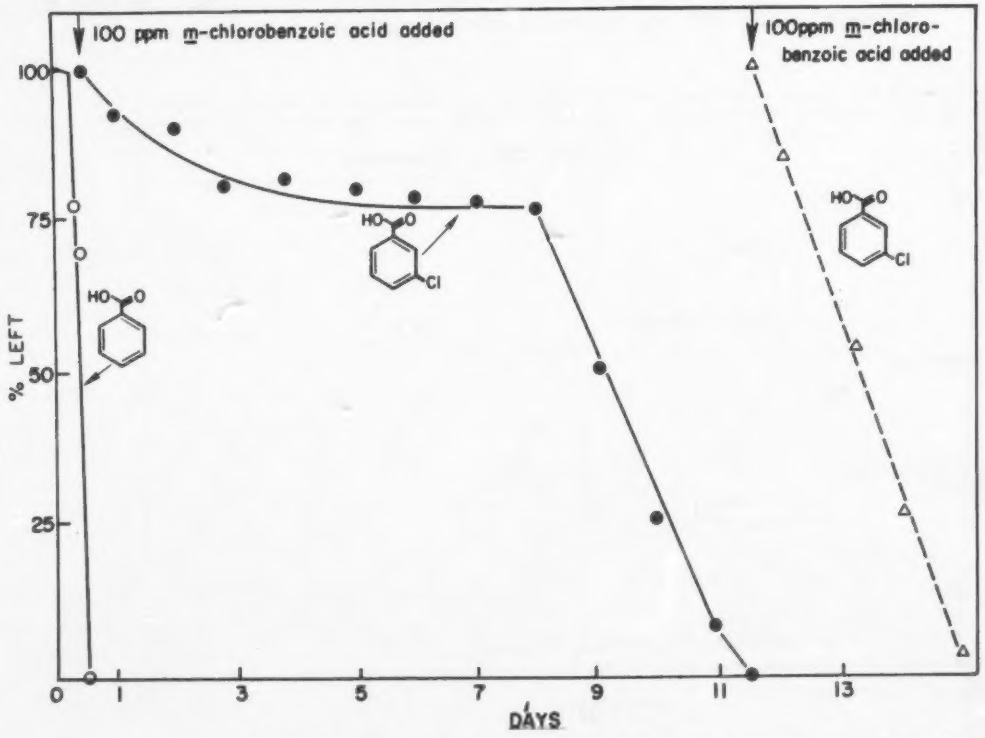

Figure 5. The degradation of *m*-chlorobenzoic acid in sewage pre-adapted with benzoic acid.

Studies were conducted to determine the effect of low substrate concentration on the degradation of *p*-chlorobenzoate and other organic compounds. The procedure involved measuring the production of $^{14}CO_2$ from ^{14}C-labeled compounds in a stream die-away system. The production of $^{14}CO_2$ from *p*-chlorobenzoate was measured at four initial *p*-chlorobenzoate concentrations (Table 1). The data are expressed as the percent of the initial ^{14}C-labeled material recovered as $^{14}CO_2$. Little or no decomposition of the parent compound was observed in at least 12 d at 47 ppm, the highest concentration used. In contrast, at 47 ppt, *p*-chlorobenzoate was extensively degraded within 4 d. At intermediate concentrations, intermediate rates were obtained. At an initial concentration of 470 ppb, a lag of at least 6 d was evident before significant degradation occurred, but the apparent lag was approximately 2 d or less at 4.7 ppb.

TABLE 1. $^{14}CO_2$ PRODUCED FROM *p*-CHLOROBENZOATE (RING-LABELED) ADDED TO STREAM WATER

Days	Initial *p*-chlorobenzoate concentration			
	47 ppm (3×10^{-4} M)	470 ppb (3×10^{-6} M)	4.7 ppb (3×10^{-8} M)	47 ppt (3×10^{-10} M)
	% of ^{14}C recovered as $^{14}CO_2$			
4	1.2	2.0	17.1	47.6
6	1.4	3.6	33.0	51.3
8	-	-	48.0	60.3
12	5.9	79.0	-	-

The secondary amines dimethylamine, diethylamine, and diethanolamine were selected for study in part because they have been shown to be precursors for the formation of nitrosamines in model ecosystems, most of the nitrosamines being potent carcinogens. Typical results showing the production of $^{14}CO_2$ from dimethylamine-^{14}C added to stream water at four initial concentrations are given in Table 2. Extensive decomposition of dimethylamine was observed within one day at the two lowest initial concentrations. Longer periods were required at the two highest initial concentrations. Apparent decomposition to CO_2 was complete within 3 d at an initial concentration of 90 ppb, whereas at an initial level of 18 ppm, the fraction degraded did not increase significantly for at least 3 d. Similar results were obtained with diethylamine.

When stream water was amended with diethanolamine, a somewhat different picture emerged. The results of a typical experiment are shown in Figure 6. At the lowest initial concentration of diethanolamine (21 ppt), degradation of the parent compound continued at an approximately constant rate for at least 4 d; at this time, about 30% of the original compound had apparently been converted to CO_2. In contrast, at an initial level of 210 ppb, an accelerating curve for degradation of diethanolamine was obtained. At 21 ppm, the highest initial concentration, less than 5% of the original chemical was converted to CO_2 within 4 d.

TABLE 2. $^{14}CO_2$ PRODUCED FROM DIMETHYLAMINE-^{14}C ADDED TO STREAM WATER

Days	Initial dimethylamine concentration			
	18 ppm (4×10^{-4} M)	90 ppb (2×10^{-6} M)	230 ppt (5×10^{-9} M)	2.8 ppt (6×10^{-11} M)
	% of ^{14}C recovered as $^{14}CO_2$			
1	1.6	14.8	84.9	97.2
2	4.3	47.0	96.3	100.0
3	4.2	100.0	-	-
6	90.1	-	-	-

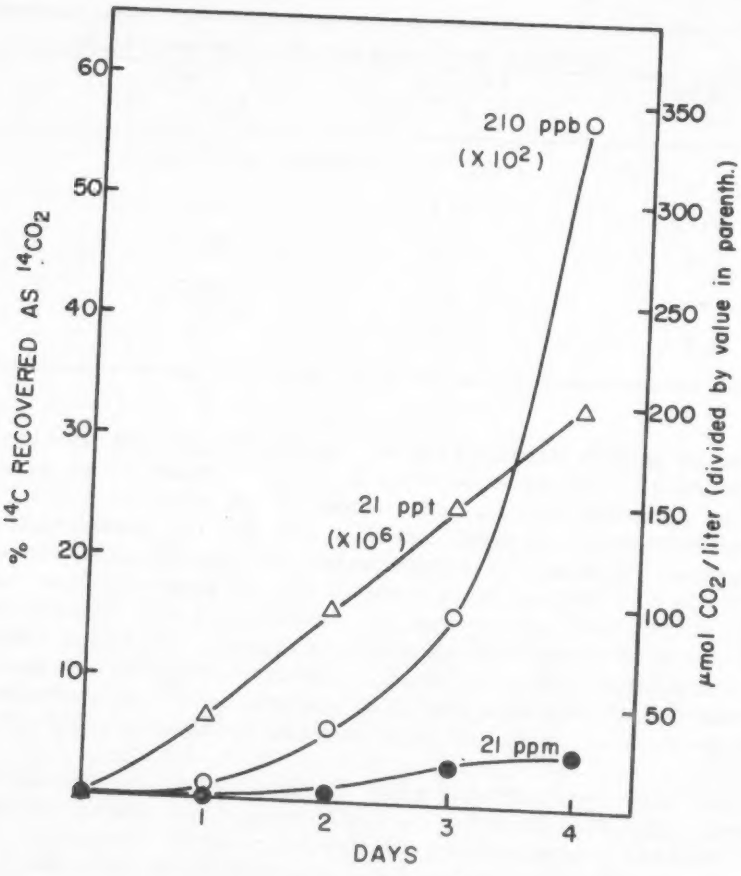

Figure 6. Formation of $^{14}CO_2$ from diethanolamine-^{14}C added at several concentrations to stream water.

The biodegradation of 2,4-dichlorophenoxyacetate (2,4-D) was similarly studied at four initial concentrations of the herbicide in stream water. The results are shown in Figure 7. The effect of initial substrate concentration on the extent of degradation of the parent compound was strikingly different from that observed with *p*-chlorobenzoate and dimethylamine. At the two lowest initial concentrations, only a small fraction of the starting material was degraded within 8 d, whereas the data indicate that 2,4-D was extensively degraded in 6 d or less when initially present at 220 ppb and 22 ppm.

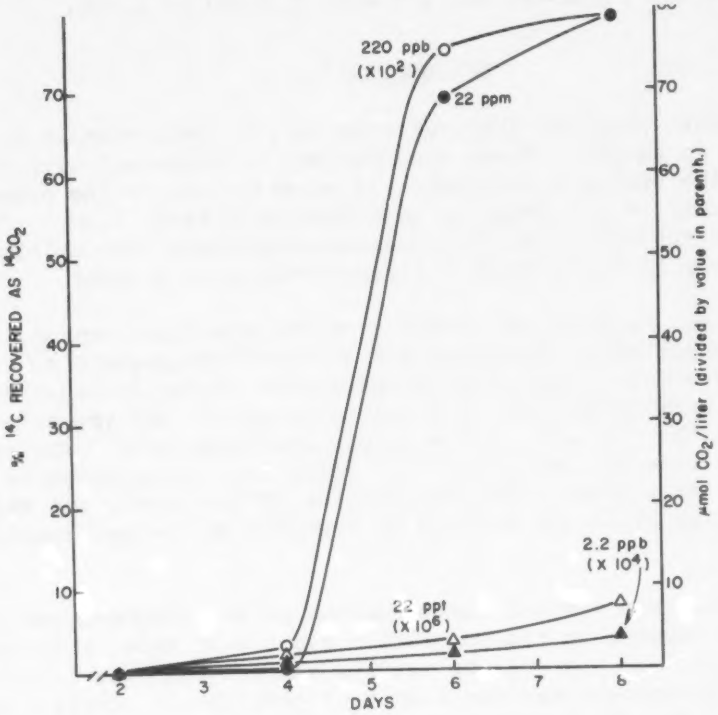

Figure 7. Formation of $^{14}CO_2$ from 2,4-dichlorophenoxyacetate (acetate-2-^{14}C) added at several concentrations to stream water.

The 2,4-D data were not the result of oxygen depletion in the sealed incubation vessels; the opposite effect of substrate concentration would be expected. The apparent absence of significant decomposition of 2,4-D at low initial concentration could not be explained on the basis of high efficiency of assimilation by the microbial population. At most, several percent of the starting material was assimilated after 7 d of incubation at an initial 2,4-D level of 2.2 ppb, as indicated by the dpm retained when particulate matter from stream water was collected on 0.45 µm cellulose-acetate (Millipore) filters and the filters counted by liquid scintillation.

Control experiments indicated that the data in Figure 7 were not the results of sorption of 2,4-D to the glass incubation vessel or to particulate matter in stream water. No radioactivity above background was detected on the filters when autoclaved stream water was incubated with 2.2 ppb of 2,4-D for 5 d and the particulate fraction collected on 0.45 μm filters. In addition, no loss of 2,4-D from solution was detected when autoclaved stream water was incubated with 2.2 ppb of 2,4-D for 36 h and the water samples centrifuged at 110,000 X g for 3 h to remove particulate matter. Dimethylamine, diethanolamine, and *p*-chlorobenzoate at similar concentrations were not detectably sorbed to particulate matter or glass in this test. Only 0.1% of the initial radioactivity was found in the fraction collected on 0.45 μm filters when nonsterile stream was was incubated for 2 d with 2.2 ppb of 2,4-D.

DISCUSSION

The data suggest that the chemical structure of chlorobenzoates affects their persistence in sewage. These findings are in agreement with published reports, in that the position and number of substituents on the aromatic ring affect susceptibility of the compound to microbial attack (1, 9). In the present data, with the exception of 3,4-dichlorobenzoate, the chlorobenzoates were more refractory if two or more chlorine atoms were present.

The data further suggest that members of the microbial community of sewage used the monochlorinated benzoates and 3,4-dichlorobenzoate as carbon and energy sources for growth. The populations active in the degradation of the mono- and dichlorinated benzoates were initially small, but the active populations increased in the presence of the usable chlorobenzoate. The apparent maximum population size was well below 10^8 cells/ml, the approximate number of bacteria expected for a substrate concentration of 100 μg/ml, but this discrepancy may be a result of the feeding by protozoa on the chlorobenzoate utilizers.

Since there was only partial disappearance of *m*-chlorobenzoate at 48 h and no detectable increase in the population capable of using this compound as sole source of carbon and energy, the results suggest that the initial disappearance of *m*-chlorobenzoate was the result of cometabolic activity by benzoate degraders. Although little is known about the role of cometabolism in degradation of chlorobenzoates under natural conditions, the ability of benzoate-degrading bacteria from various genera to cometabolize chlorobenzoates is well documented (14).

The results from studies of the effect of concentration of *p*-chlorobenzoate and dimethylamine on their biodegradation indicate that these compounds are extensively decomposed within a few days at the lowest initial concentrations and thus would not persist in freshwater at these levels. In contrast, all of the compounds studied at ppm levels, except for 2,4-D, were degraded only after significantly longer lag periods or not at all within the time of study. The biodegradation of organic chemicals in die-away systems with low microbial densities has commonly been assessed at initial concentrations of the test chemicals in the ppm range, but these data demonstrate that many chemicals that appear to be persistent in surface waters at ppm concentrations may be rapidly destroyed at lower levels. This effect of concentration must thus be taken into account in designs of test systems for evaluating biodegradability.

These findings also suggest that diethanolamine and 2,4-D may be more persistent at very low than at higher concentrations. Thus, low substrate concentration may also limit biodegradation rates in natural waters. A possible explanation for the linear increase with time in the rate of degradation of diethanolamine at the lowest initial concentration is that the population active in its degradation does not increase significantly at the expense of diethanolamine at this level of substrate. This interpretation suggests that organic chemicals may be present at such low concentrations in waters that the rate of microbial decomposition is limited by the initial size of the active population.

Although the conversion of part of the ^{14}C-labeled material to volatile metabolites other than $^{14}CO_2$ cannot be excluded, the data show that extensive degradation occurred. The approximate yields of CO_2 from the various organic compounds were generally consistent with expected values, and experiments in which ^{14}C-glucose was added to stream water at several initial concentrations gave values of 50-60% recovery of ^{14}C as $^{14}CO_2$.

Our findings point to the possibility that many organic pollutants may persist in aquatic ecosystems owing in part to their low prevailing concentration. If persistence is sometimes a consequence of the inability of microorganisms to destroy biodegradable molecules at low concentrations, this failing may account for the presence of trace levels of certain organic chemicals in natural waters. Such trace substances could lead to environmental problems if they are susceptible to biomagnification and are subsequently toxic to species at higher trophic levels in food chains. Furthermore, recent studies have suggested that some organic chemicals may be toxic to aquatic microorganisms at ppb levels and lower (5, 10). Hence, further inquiry is necessary to define more precisely the influence of concentration on the rate of microbial destruction of organic molecules in aquatic ecosystems.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Alexander, M. 1965. Biodegradation: problems of molecular recalcitrance and microbial fallibility. Adv. Appl. Microbiol. 7:35-80.

2. Alexander, M., and B. K. Lustigman. 1966. Effect of chemical structure on microbial degradation of substituted benzenes. J. Agric. Food Chem. 14:410-413.

3. American Public Health Association. 1976. Standard methods for the examination of water and waste water, 14th ed. American Public Health Association, Washington, D.C.

4. Arnow, L. E. 1937. Colorimetric determinations of the compounds of 3,4-dihydroxyphenylaminotyrosine mixtures. J. Biol. Chem. 118:531-537.

5. Batterton, J., K. Winters, and C. Van Baalen. 1978. Anilines: selective toxicity to blue-green algae. Science 199:1068-1070.

6. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1: 279-285.

7. DiGeronimo, M. J., M. Nikaido, and M. Alexander. 1978. Most-probable-number technique for the enumeration of aromatic degraders in natural environments. Microbial Ecol. 4:263-266.

8. Jannasch, H. W. 1967. Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol. Oceanogr. 12:264-271.

9. MacRae, I. C., and M. Alexander. 1965. Microbial degradation of selected herbicides in soil. J. Agric. Food Chem. 13:72-76.

10. Powers, C. D., R. S. Rowland, H. B. O'Connors, Jr., and C. F. Wurster. 1977. Response to polychlorinated biphenyls of marine phytoplankton isolates cultured under natural conditions. Appl. Environ. Microbiol. 34: 766-764.

11. Ryckman, D. W., V. S. Prabhakara Rao, and J. C. Buzzell, Jr. 1966. Behavior of organic chemicals in the aquatic environment. Manufacturing Chemists Association, Washington, D.C.

12. Shehata, F. E., and A. G. Marr. 1971. Effect of nutrient concentration on the growth of *Escherichia coli*. J. Bacteriol. 107:210-216.

13. U.S. Environmental Protection Agency. 1971. Algal assay procedure-bottle test. U.S. Environmental Protection Agency, Corvallis, Oregon.

14. Walker, N., and D. Harris. 1970. Metabolism of 3-chlorobenzoic acid by *Azotobacter* species. Soil Biol. Biochem. 2:27-32.

DISCUSSION

TRANSFORMATION IN AQUATIC ENVIRONMENTS

CLESCERI: Dr. Floodgate, is it possible that the organisms can use some of the organic nitrogen in the oil itself? There is a certain amount of heterocyclic-combined nitrogen in oil. Is this unavailable to the organism?

FLOODGATE: Yes, more or less. One or two organisms have been shown, in batch culture, to be able to use the nitrogen in this cyclic material, but only in warm temperatures. In other words, in nature, there are other substrates available for them to metabolize.

CLESCERI: Do you think that means, then, that these compounds are permanently nondegradable?

FLOODGATE: Most of them are nondegradable, or at least with a very long turnover time, perhaps decades.

JANNASCH: Dr. Wright, you presented a slide showing the decrease in specific heterotrophic activity starting up in the marshes of an estuary and moving toward offshore waters. I was wondering if you thought that these results might be due to some effect of the increase in salinity which decreased specific activity as opposed to just a decrease in nutrient availability.

WRIGHT: The range in salinity in the estuary is not great; it's a very low fresh water input estuary. The range of the salinity might be a total of 5%; I don't think that could be a very serious problem.

JANNASCH: In the acridine orange stain, does that stain both viable and dead cells, or primarily viable cells?

WRIGHT: It stains any cell that has nucleic acid in it. Dr. J. W. Costerton thinks that there are hardly any dead cells in natural waters. They die, autolyze, and are gone pretty rapidly. But, then, how do you define viability? Do you define it as whether you can count them on agar plate? If so, then a very small percentage are viable. If you count them on the basis of what they are doing, in terms of activity, then a large percentage are viable.

P. ROGERS: You mentioned that Beta bacteria were capable of utilizing excreted soluble organics. I was wondering, do you think bacteria are capable of actually lysing algae or at least attacking algal detritus material directly through extracellular enzymes?

WRIGHT: I'm not too familiar with this work but I think that people who have examined healthy algae under the microscope from sea water and fresh water rarely find bacteria on their surfaces, unless they are blue-greens that have large slime sheaths. I haven't come across research that shows bacteria attacking algae and trying to release the organic compound from them.

ROGERS: I feel that this is a viable alternative, and I have photographs of kinetic data showing that this is a possibility. I believe that plate counts, for many applications, is definitely a viable alternative to represent heterotrophic activity. I think some of your graphs may have indicated that where you have no increase initially in your total counts, but plate counts and other methods of indicating heterotrophic activity showed a linear response immediately to confinement in the bottle approach. Am I mistaken?

WRIGHT: I don't understand what you are saying.

ROGERS: I think that you are counting a lot of bacteria that are not viable at that time or never will be viable, at least in fresh water systems. Recent work by Jones shows this to be true.

WRIGHT: How do you account for the large number that eventually show tetrazolium salt reduction, up to 70-80%? Other people have shown with autoradiography close to 100% of the bacteria developing on the film, indicating activity.

ROGERS: Sea water system or fresh water?

WRIGHT: Sea water.

PASSMAN: George, you mentioned that under low nutrient conditions, you had ATP pools that were too low to provide for initiation of enzyme synthesis, or growth, necessary to begin incorporation of hydrocarbons. Have you looked at the cyclic AMP pools?

FLOODGATE: I was told to be a little controversial or stir things up; that was material I threw in without the information, as yet. What happens when you get a job to do like this, you find yourself writing down your research program for the next ten years. That's one of the items on it. I want to go back now to see what actually happened to that nitrogen. Remembering the graph, it does disappear into the cell somewhere; we have not looked at it closely; it just disappears. We've assumed it goes into the cells because Charlie Brown has shown that it does go into their *Pseudomonas*. We have yet to show that it goes into our oil-degrading "bug." Whether it actually goes into the glutamate pool, whether it stays in there or comes out again, or what happens, we just don't really know yet.

PASSMAN: Dick [Wright], it was interesting to see that your specific activity increased under conditions where nutrient levels were higher as we were discussing yesterday. Looking at LPS to direct count ratios in the Georges Bank, there were different patterns. During the winter the LPS to acridine orange direct count ratios were low but when we got into the growing season, the LPS per cell apparently increases. We also saw this type of phenomenon in dump sites where hours after drug residues were pumped into Puerto Rican Harbor there was a rapid decrease in the LPS per cell suggesting that we may be looking at a parallel type of phenomenon here that would be worth further consideration.

RAYMOND: Dr. Floodgate, I missed one of your rate uptake numbers. You said 30 grams of carbon or oil, is that per cubic meter of seawater per year?

FLOODGATE: One cubic meter of seawater provides sufficient nitrogen to degrade 30 grams of oil in the summer, per year, or 11 grams of oil at winter temperatures, per year.

RAYMOND: I would like to throw this out for the audience since we have a lot of marine microbiologists and chemists here. Let's talk about mass balances. Don't we have a much larger organic turnover, mass balance, which is not reflected in terms of pollutant turnover? What amount of material do we have turned over in marine waters?

FLOODGATE: The figures are much higher for natural material. I think it is 10^{18} grams of carbon annually in the ocean. The amounts of pollutants are many orders of magnitude less. So for total heterotrophy in the ocean, man's effect is insignificant. But most of our rubbish is not chucked into the entire ocean. We do not take garbage from London to the Pacific Ocean; we chuck it into the English Channel. This is also an area where we have a lot of fishing and oysters. This is highly productive area. I don't think we have begun to approach one of the real problems: these areas are used for two opposing purposes--as a source for food and recreation and as places to dump our rubbish. These two ideas are really in opposition. The North Sea has been used as a dump for rubbish from the British northeast industrial complex around Newcastle. All around the North Sea there is heavy industrialization and even before we were aware of the environment, we had been dumping stuff in there. The North Sea is still one of the world's richest fishing grounds. Does it really matter? Do we need all these kinds of regulations either from EPA or from our side of the European pond, the Commission in Brussels?

RAYMOND: I think an important point to consider is the type of system we are using and the methodologies to evaluate whether a compound is degraded. We have to select a system and we should not select the very low carbon seawater.

LITCHFIELD: Dr. DiGeronimo, do you have any idea of the in-situ or natural concentrations one might expect to find of the *p*-chlorobenzoate or dimethylamine?

DiGERONIMO: Well, in our sewage systems some of the effluent compounds are probably found in high concentration. We felt the concentration in the sewage may even have been below realistic values seen coming out of a factory. In natural waters there is a constant dilution of the compound and an immediate dilution as it enters from a point source.

LITCHFIELD: This is providing a carbon source or nutrient for the environment, particularly in the river system, so you might expect to find these rates of degradation. In terms of bacterial decomposition rates, are you overloading the river system or is the concentration below what the organisms might detect, or what?

DiGERONIMO: I don't think I want to say anything about rate. I'll talk in terms of persistence. I think we showed a trend and naturally more and better rate data are necessary to confirm our systems.

JOHNSON: Could you elaborate on the most probable number approach you're using in terms of the method, a three-tube or five-tube system, and some of the statistics? I think many of our problems involve an effort to quantitate biomass.

DiGERONIMO: You are absolutely right, but I think anybody who tries to justify a population study without substrate disappearance would be in trouble. We developed a system for the aromatic degraders to try to quantitate a particular population within a larger population. As the data showed, our populations of benzoate degraders in sewage were always found over the year at 10^6/ml. To quantitate a population that is found at 10^2/ml, and only reach 10^6/ml would be virtually impossible to observe and the types of population shifts that were taking place. To answer your specific question, we did use a five-tube MPN method.

CHAPMAN: Do you have any information as to the bacterial genera which turn up in your experiments, with a capacity for growth with chlorobenzoate?

DiGERONIMO: Yes, but only that they are gram-positive or gram-negative.

CHAPMAN: In other words, you've not made generic assignments?

DiGERONIMO: No.

CHAPMAN: This is of interest, of course, because the question arises, if, for example, your organisms were from the genus *Arthrobacter*, would you logically expect those organisms to be selectively enhanced in their population by the addition of benzoate or would benzoate simply increase the population of many other organisms, lacking the ability to grow at the expense of chlorobenzoate?

DiGERONIMO: You are talking about what would be the species of the population?

CHAPMAN: I think it is of importance to identify certain of these genera, to know something about their division times in these mixed cultures, and to anticipate whether benzoate might logically be expected to increase their numbers.

DiGERONIMO: I agree.

TIEDJE: Last night we had a good discussion on cometabolism, but one aspect we haven't really considered, related to cometabolism, is uptake. Normally we think of cometabolism in terms of degradative enzymes, not of transport enzymes. Presumably with low concentrations, we could see a transport system which would be non-specific. I'm wondering if one could obtain useful information about cometabolism with low concentrations by simply studying the kinetics of uptake of the particular compound and its co-substrates. Have you given this any thought? For example, what is the K_t of uptake of the chlorobenzoate compared to benzoate?

DiGERONIMO: We have not addressed transport to confirm that. However, there can be an extracellular or surface enzyme for transformation of the compound.

TIEDJE: Most of those studies have been done at higher concentrations where, presumably, you could have a non-active uptake system. If you are talking about low concentrations, as would be expected in effluent systems, you are going to have to concentrate against a gradient. Presumably there is a benzoate transport system that is also taking in chlorobenzoate. If you knew the K_t for chlorobenzoate transport, then you would be able to predict at what concentrations you would get uptake or not.

BOLLAG: When you pre-culture the organisms on the substrate benzoate, is there a change in the transformation ability of the microbes at different concentrations?

DiGERONIMO: For low substrate concentrations, we haven't worked with pre-adapted systems. I don't know whether pre-adapted cells attack lower concentrations differently.

PASSMANN: Dr. Blackburn, your data on ammonia production versus utilization, in the first season's graph, showed tremendous utilization but you still have substantial ammonia in the sediment. Also you mentioned that in the deeper sediments you see some curvature rather than a straight increase in the amount of ammonia. How precise is that data and, at one particular spot, is that ammonia deficit, real, or is it simply an anomaly of one observation?

BLACKBURN: No, there is a certain amount of scatter in this sort of data, but over the five stations we've studied this is a definite trend. When we see a dip in the ammonia profile, this is associated with net uptake of ammonia. There is a depletion in the ammonia pool rather than an increase in the ammonia pool.

PASSMAN: At one station there seemed to be a fairly substantial ammonia pool but a much more pronounced utilization than any of the other stations in that figure.

BLACKBURN: One measurement is in static pools and the others are rates, so they are in different units. I'm not quite sure I understand your question.

PASSMAN: The question I was asking was, at that particular station was that a real phenomenon, is there enough replication there to say that there was a real utilization of ammonia vs production?

BLACKBURN: This is right. We see the same trend in the other five stations we examined, but it is not absolute. Sometimes the gradient that one sees is the result of what has gone on in the month before. The rate is the result of what is going on now. So there is a certain time lag before the gradient changes to the rates that are operating at that point in time. So if you want to try to analyze it you can say, the rate before was such and such, the rate after was such and such, and the rate now is this. But you don't know quite how the rates have operated in between those times. It may have been steady up to the day before and something changed. It is somewhat difficult to take the kinetics and explain absolutely the static data. In general, there is a correlation, but the whole thing is confused because the gradients themselves are mixed up by perturbation, in effect, the whole gradient may be moved upward.

PASSMAN: Thank you.

COLWELL: Dr. Floodgate is looking too relaxed; I should like to elicit a comment from him. In your discussion of oil degradation, you pointed out the requirement for nitrogen in the degradation of certain amounts of oil, but in a previous publication of yours, you made a very important statement that oil spilled during World War II could still be detected in the Atlantic Ocean. What were you measuring? Isn't it a fraction, a portion of the oil that is a residual portion, namely the resins, asphaltinic, and aromatic compounds which are really not being attacked in the short time frames of most studies? Could you make some sort of statement about this part of the oil that probably poses more of a problem than the short-chain alkanes which everybody, by now, knows are readily degraded?

FLOODGATE: Of course, you are absolutely right. The large ring compounds certainly have a very long turnover time. I imagine that these can be in the order of decades or even longer. One tends to think of oil as one substrate when, of course, it is many thousands of different substances. In fact, if I remember right, there are something like 200 to 300 *n*-alkanes alone. I don't think we can say very much about the rate of degradation of these very resistant materials. One can say they seem to stay on the bottom, because I think most of them seem to end up in the sediment and one gets the impression they are going to remain there for a very long time. It could be decades, it could be centuries in certain situations. Whether one should be bothered about this is another matter; after all if they are not going to be attacked by bacteria one gets the general impression they are more or less biologically harmless because they are always covered by water, so they are not going to cause any immunity problem. They are just sitting there on the bottom; they would eventually presumably be covered up. One can just hope that they stay there long enough until some future scientist in the year 2500 or something comes along, digs them up and has another look at them. But I don't think they need bother us. They are just there, and they are going to stay there.

COLWELL: I can accept part of your statement, but not all of it. What about the polynuclear aromatics being taken up by the food chain, as some studies are beginning to show?

FLOODGATE: Yes, these are the photo-intermediate ones, aren't they? They possibly are the ones that we should be worrying about a bit more.

COLWELL: They are possibly carcinogenic.

FLOODGATE: Some of them are supposed to be carcinogens. But there is no evidence, as far as I know, that any of them are causing any trouble. The amount of carcinogen in oil is very low, a few micrograms/100 tons. Some of them get into the food chain, some of them probably get into us, but probably we pick up fewer carcinogens from oil than from walking down Main Street or Fifth Avenue. So should we be bothered about it?

COLWELL: I am asking you.

FLOODGATE: I'm not worried about it.

ALEXANDER: Relative to some of the data that Dr. DiGeronimo presented, it is important from the viewpoint of the workshop and regulatory agencies to point out the marked effect of concentration on the rate of decomposition. Although the data presented were in percent of the substrate destroyed and converted to CO_2, since the concentration declined by orders of magnitude, it meant the rate also declined by orders of magnitude. In going from a test system using parts per million to a real environment with parts per billion or parts per trillion levels, then we have to assume a decrease in rate by 10^3 or 10^4 (or more) fold. Conversely, at very low substrate concentrations, as indicated in the case of 2,4-D, essentially nothing happens. This is apart from the conventional Michelis-Menten kinetics. H. Jannasch, A. G. Marr, and others have demonstrated this in pure culture, but our studies dealt with an effect in a natural ecosystem. The question I have relates to some of the comments of George Floodgate. George, looking at the issue from the viewpoint of the regulatory agencies and extrapolating from a model to a natural environment, what do you feel the effect of particulates would be on the biodegradation of the non-oil pollutant in the marine environment? Is a water-soluble substrate sorbed to a particle--more readily decomposed, for example, because of the higher concentration? Or would it be more slowly decomposed because of the problems of desorption or the need for breaking down some type of bonding?

FLOODGATE: I think that might be explored. We've been studying urea, and certainly it can be absorbed onto clays. The molecules being small can get into the interstitial part of clay where the bug cannot get at it. So to that extent, clay protects urea and it will stay there forever. On the other hand, it can get onto the outside by ionic absorption or Van de Wouls absorption and it might build up to a level where a bacterium has got something to go for. But I think the answer is both, it depends on the material it is absorbing and also the absorbent material.

ALEXANDER: Have you done any work with any other compounds than the low molecular weight material which might get into the lattice structure of the clay or otherwise absorbed?

FLOODGATE: No, we haven't, but I believe some people have and have seen the same kind of thing.

ALEXANDER: In the marine environment?

FLOODGATE: Yes, I think so.

SOMERVILLE: First of all, I would like to thank Dr. Floodgate for acting as an apologist of the oil companies. I would like to reassure people that certainly Shell is concerned that potentially harmful compounds have formed during the natural degradative process, when oil is released into the environment. Also, I would like to point out that there is a great deal of natural seepage of oil. I have two questions, first of all for Dr. Floodgate, concerning oil spills. Is there any evidence when you get a massive contamination with primarily a carbonous energy source, that this acts as a reservoir for nitrogen fixation which then acts in an autocatalytic way to promote degradation?

FLOODGATE: I don't think so. More nitrogen fixation in the ocean is done by an algae, a green algae. I don't think there is any evidence that the fixation which is undoubtedly carried out by marine bacteria--Clostridia, Desulfovibrios--is sufficient in quantity to make a really big difference. There are reports of hydrocarbons being the carbon source but I don't think that a big oil spill is going to encourage much of this fixation. We have been looking recently at the effect of oil on algae. We find that small amounts of oil tend to stimulate algal growth and large amounts tend to decrease algal growth. Now whether this will be the same kind of thing for the blue-greens which are the nitrogen fixers, I don't really know. I think I would like to apply to Shell for a grant.

SOMERVILLE: There is a lot of circumstantial evidence for this. Large spills on land, for example, do promote nitrogen fixation.

FLOODGATE: True enough, that work has been done by the people at Westbury; it happens on dunes and possibly salt marshes but I don't think it happens in the open ocean. The nitrogen fixation mechanisms are quite different, and we haven't anything in the sea that corresponds to *Rhizobium* or *Azotobacter* in the ocean. These are available in the marshes in the plant life. It does go on in the land but not in the sea.

SOMERVILLE: Thank you. The other question I have is for Dr. Kadota. It refers to his limitation of growth rate as a function of carbon concentrations. I think that both of your cultures reach a max at about 20 mg/liter carbon. A quick calculation suggests that this is approximately equivalent to the source of nitrogen concentrations we have been talking about and suggests that it may not be just nitrogen limitation which is limiting growth. Do you know if growth is limited by anything else or do you have any similar data with nitrogen?

KADOTA: I have no data on the nitrogen limitation on the program.

PRITCHARD: Dr. Wright, I am concerned about effects on the properties of the systems that you see due to bottling or "containerization." My question, basically, is, do you think we can settle on a set of conditions or time to study the degradation processes in a system? For example, do we study degradation based on the 0-12 hours or 0-2 days incubation; or do we wait 12 days and then examine the system for degradation?

WRIGHT: What we are looking at is a continuous process. During the first few hours, I think the response is probably very close to the natural in-situ response. During the first day, there is adaptation and some growth occurring. After the first day, I think the population is being selected, and selective effects are going to probably take precedence over any natural effects. So if you want to set hard rules or procedures, you must limit it to 12-24 hours for natural rates, depending on the temperature--the colder the temperature the longer the incubation time. For a fairly natural population response you might incubate as long as two days. Beyond this, you obtain a highly selected response of a highly selected population.

PRITCHARD: Can I go to the extreme and say if my compound won't break down to any great extent in two days, I could extrapolate to a persistence in a marine environment?

WRIGHT: I think one would have to be very careful about that extrapolation. I would suggest there are probably other processes involved such as absorption to particles, which will provide for breakdown of a type we don't see typically in planktonic bacteria. I don't know the answer to that.

LITCHFIELD: A question related to this, to what extent to you re-oxygenate the water, or is this not a consideration when incubating a closed system for 24-48 hours or longer?

WRIGHT: This is not a problem. I've been dealing with really quite oligotropic systems.

LITCHFIELD: You have no oxygen deficit?

WRIGHT: No.

SLATER: Dr. Kadota, have you done any experiments in your continuous enrichments at two concentrations of substrate, where you incubated them for a longer period of time than the relatively short time on the data you presented, I wonder if the composition of the two communities changes in these open growth systems?

KADOTA: I have not incubated for long periods, so I have no data on such effects.

PASSMAN: Dr. Wright, over the past couple of years there have been a number of studies in which dialysis was used to incubative systems in situ. Would you predict or do you have any information to suggest that wall effects would be of the same magnitude in these systems as in your systems?

WRIGHT: I've never used them; but my guess would be that the same things would happen, perhaps greater because you would probably have leaching of substances from the dialysis tubing itself.

IVANOVICI: Dr. Blackburn, I think that developing a method for estimating the health of a system is very important. You said that you had done most of your work in a non-polluted system. I would like to know if you are going to test your hypothesis in a polluted system, and if so, what sort of predictions you might make about the ammonia levels and the other parameters you are measuring. I would expect you would be able to make predictions for such a method.

BLACKBURN: Well, it isn't absolutely a clean system, it's a brackish fjord that cuts across northern Denmark. There probably are inputs from cities, etc. The problem is, you don't know exactly what the inputs are, and this is part of the reason we adopted this approach of determining what is happening in the sediment and what's getting out of the sediment. We can, in fact, determine what's getting into the sediment. It isn't easy to know exactly what goes in. The prediction I would make, if there is an increase in input into the sediment then all the rates of turnover should increase and one should be able to determine the change. If hydrocarbons, cellulose, or something with a high carbon:nitrogen ratio gets to the sediment, then one should immediately see this effect. If it is breaking down then one should get an increased uptake of ammonia; if it isn't breaking down then one should see an increased total organic content in the sediment. It all depends on quantity; and this again comes into what is your definition of pollution and if you base it on quantitative or qualitative considerations. These are the sorts of things one might see depending upon what you are putting into the system: increased rate, increased organic compounds, or changes in the calculated nitrogen: carbon ratio.

IVANOVICI: Do you think that you would have some range of values for rates that you might associate with an unhealthy system?

BLACKBURN: No, not really. Because we have a range of sediment types, some have a higher organic input than others, and they are turning over. It's really a matter of defining what parameters can describe the system, then, if it is perturbed in some way one should see a change of those rates or parameters. I wouldn't like to say that a certain rate indicated health. It indicates normality, and some change indicates abnormality.

IVANOVICI: I would like to direct a question to the panel as a whole. We've had presented here a discussion of the effects of a number of different pollutants. I think it's really important in such a workshop that we define certain terms. Can you put forth a definition of pollution? What criteria are you using to define pollution, perhaps concentrations of the various chemicals, or in terms of biological effects, or in terms of nutrient levels and nutrient effects?

FLOODGATE: All I can do is recommend that you come to my lectures. Lecture No. 2 is on definitions of pollution, and there are five or six completely different definitions. I try to use this approach to show the student that pollution is what you want it to be. Now we are here with reference to the EPA and regulatory agencies in the United Kingdom, and the definition accepted is one agreed on by the FAO in Rome in December 1970. I can't quote it verbatim, but it is roughly that marine pollution is anything that fouls up the environment from the human point of view. That is, if it destroys amenities, interferes with fishing, interfers with quality of water from any human angle. This is a view of a natural environment which many biologists, the environmentalists, find very unpleasant; they don't like it at all. They feel that what promotes human well being is good, but that isn't the same thing as what promotes the well being of the natural environment, the natural creatures. So there are other definitions of pollution which take into account the creatures that live in that environment. There are others which are based on information theory; there are others aimed toward one particular group, one is more microbiological in point of fact. So the trouble is you are trying to define something which literally cannot be defined. It doesn't fit into a logical category of definable objects, because you can't make limits on these things. Something like the FAO proposal is the best you can do as a practical working guide for regulatory organizations like the EPA.

IVANOVICI: Is that the definition that is being used here, then?

FLOODGATE: I don't know what definition the EPA uses, but this is the one that GESAMP (Group of Experts on Scientific Aspects of Marine Pollution) uses. In fact, as I remember, the FAO definition has several categories. One is impairment of fishing, another is quality of water, and the third is impairment of human activities which means things like swimming, etc. They list each of the problems under these headings, so a particular substance going into the sea will be given a high rating under one and a low rating under another heading. They are probably good working definitions, but they don't suit the environmental group, they don't like it at all.

IVANOVICI: I was just curious.

STEWART: Dr. Wright, isn't it possible that in the acridine orange direct count method, you are getting or could get population shifts, without materially altering the total number. What assurances do you have that this does not take place?

WRIGHT: It's very possible. We just don't know; it's possible they are still dying and new cells dividing, adding to the population without a total change. We just don't know at this point. There hasn't been enough work with that method. Is that what you were referring to?

STEWART: Yes.

FLOODGATE: This fluorescence method is a very interesting one. Did you say you were using formaldehyde, I think it is a preservative? We've been adding glutaraldehyde, because it does less damage to the more delicate bacteria giving slightly high numbers. So I suggest you try glutaraldehyde.

WRIGHT: I've tried it and encountered real problems with flocculation. It seems to create a lot of heterogeneity on the filters. That's just in our laboratory.

YOUNG: Dr. DiGeronimo, in our tertiary-treated waste water, we find mono-, di-, and trichlorobenzene, not the benzoate. I'm wondering whether you have any data on decomposition on these mono-, di-, and trichlorobenzenes.

DiGERONIMO: No, but in working with engineers at Cornell, we were asked a rather similar question. We showed degradation and adaptation of sewage, but it took three days to get rid of the substrate. One should consider that some compounds from industrial effluents may have a residence time in a sewage treatment plant of only 24 hours. In 24 hours, only 50% of the compound is actually being acted upon, and what one sees reaching the environment is the other 50%. So, you may have to consider a longer residence time to get complete destruction of some xenobiotics.

YOUNG: Regarding chlorobenzenes, would you anticipate any particular problem in their decomposition?

DiGERONIMO: This is speculation, but I would say no. When I first started, I thought I was going to get little degradation of several compounds, and I started finding degradation.

COMPARTMENTALIZATION IN AQUATIC ENVIRONMENTS

Chairperson, Gordon Chesters

THE MARINE ANOXIC ENVIRONMENT

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ABSTRACT

Anoxic conditions are rare in seawater but where anoxia does occur it is usually because of restricted water circulation and hence oxygen diffusion, as for example, in certain fjords, deep trenches, estuaries and landlocked bays. The physical restriction of oxygen supply is often combined with an increased microbial oxygen demand resulting from the presence of large quantities of utilizable carbon, e.g., a senescent algal bloom, domestic sewage or industrial effluent. In the sediments of the continental shelf, however, the situation is different for with the exception of the so-called "oxygen" or "high-energy windows" the thin surface oxic zone overlies an extensive anoxic zone. Considerable mineralization can occur in the anoxic zone; for example, Jorgensen (10) calculated that 53% of all the organic carbon mineralized in the sediment of a Danish fjord was catalyzed by the anaerobic sulphate-reducing bacteria. In shallow estuaries receiving industrial effluent the microbial activities in anoxic sediments have been shown to be a major factor in the development of a "pollution syndrome" (1, 19). This paper is concerned with the anoxic compartment of the marine environment, in particular its development, and biological, chemical and physical characteristics. The maintenance of the anoxic condition and the relationship between the anoxic and oxic systems is examined.

BIOLOGICAL CHARACTERISTICS OF AN ANOXIC SYSTEM

There are three processes by which an organism can generate energy in the absence of oxygen, i.e., fermentation, bacterial photosynthesis or anaerobic respiration (see 8, 13, 23).

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In fermentation, organic compounds serve both as primary electron donors and terminal electron acceptors. Some organisms have to be supplied with separate sources of electron donor and electron acceptor, for example, *Clostridium kluyveri* requires acetate plus ethanol; however, other organisms can obtain both electron donor and electron acceptor from a single compound. When compared with aerobic respiration fermentation is marked by: a) a comparatively low yield of energy and hence of growth per mole of substrate consumed (the cell yield is generally below 10%) and b) the formation and excretion into the surrounding medium of "energy-rich" fermentation products, often in amounts equimolar with the substrate being fermented.

Except for the cyanobacteria, bacterial photosynthesis is a totally anaerobic process and oxygen is not produced by the photolysis of water. The photosynthetic bacteria are therefore found in anoxic environments, where there is sufficient light energy and suitable electron donors, e.g., sulphide.

In anaerobic respiration an electron acceptor other than oxygen has to be used. The principal acceptors are nitrate, nitrite, fumarate, sulphate and CO_2. In nitrate respiration the nitrate is reduced to nitrite. This reaction is widespread among facultative anaerobic bacteria belonging to a number of genera, including *Pseudomonas*, *Bacillus*, and various members of the Enterobacteriaceae. In denitrification the nitrate is reduced through nitrite, nitric oxide and nitrous oxide to nitrogen and even ammonia. This process is confined to a more limited group of facultative anaerobic bacteria (17). Fumarate reduction is effected by a broad range of bacteria (11) as well as in some protozoa and helminths.

Bacteria which use sulphate or CO_2 as terminal electron acceptors show a number of significant differences from those able to grow anaerobically with nitrate or fumarate. They are strict anaerobes, their respiratory mechanisms can only be coupled to sulphate or CO_2. They also have a restricted nutrition since only a limited range of compounds can serve as electron donors. Bacteria capable of coupling substrate oxidation to the reduction of sulphate (or in some instances, sulphite, thiosulphate, tetrathionate or sulphur) to sulphide, are members of the genera *Desulfovibrio*, *Desulfotomaculum*, *Desulfomonas* and *Desulfuromonas*. The nutrition of these organisms is limited to the oxidation of hydrogen, pyruvate, lactate, ethanol, formate or malate which are converted to fatty acids (acetate) and CO_2. *Desulfotomaculum acetoxidans*, however, has recently been reported as capable of utilizing acetate (25). Bacterial sulphate-reduction in anoxic marine systems is of considerable importance because of the high sulphate concentration in sea water; in marine sediments sulphate can be detected at depths of one meter or more. In the absence of sulphate these bacteria are capable of fermenting ethanol or lactate to acetate and hydrogen. This process will only take place, however, if the partial pressure of hydrogen remains low, as would occur if hydrogen-utilizing bacteria are also present in the system.

The methanogenic group of bacteria are strict anaerobes which obtain their energy by coupling the oxidation of a very limited range of nutrients to the reduction of CO_2 to methane. The principal electron donors are hydrogen and acetate, although methanol and formate can also be used; the latter is, however, generally first cleaved to CO_2 and hydrogen.

It is often thought that the anoxic marine environment is the sole preserve of obligate and facultative anaerobic bacteria. For many anoxic systems, this is an erroneous view as was demonstrated by Fenchel and Riedl (6) who found both ciliates and other invertebrates active in anoxic systems.

CHEMICAL AND PHYSICAL CHARACTERISTICS OF ANOXIC SYSTEMS

The methanogenic bacteria require more reduced conditions than the sulphate-reducers for growth, which in turn require more reduced conditions than the denitrifiers. Therefore, as a system goes from the oxic to the anoxic condition a succession of organisms utilizing anaerobic respiration occurs. By measuring the redox potential of a system it is therefore possible to determine which electron acceptor is being used in anaerobic respiration. Such a succession in both the microbial population and the redox potential is shown as a function of sediment depth in Figure 1. This is an idealized situation since in many sediments all the zones are not distinguishable because of factors such as a low nitrate concentration and the activities of burrowing animals. Also, Figure 1 is a human or "macro-organisms" view of the sediment, and it does not recognize that microbial activity occurs principally within microenvironments. It is therefore to be expected that, for example, denitrification or sulphate-reduction (9) will occur in what on a gross scale would be called an oxic environment. There is little information on the role of such anoxic microenvironments in mineralization, since the majority of our techniques (and philosophy?) are designed to determine the average chemical and microbial characteristics of a large number of microenvironments. This criticism can, of course, also be applied to techniques for determining the redox potential of a sediment. Since the platinum electrode behaves reversibly under anoxic conditions it is relatively simple to determine the redox potential of a sediment. However, there are some major interpretational and methodological problems with these measurements (16, 24), for example, which redox couple is actually dominating the system, the problem of inserting the probe without mixing oxic and anoxic zones and the need to prevent interference caused by contamination of the platinum proble or reference electrode. Nevertheless the redox potential, especially when coupled with other biological and chemical measurements, provides a useful characterization of the system.

Bacterial sulphate-reduction, and hence sulphide production, is an important reaction in marine anoxic systems. Sulphide is also produced by the degradation of sulphur-containing amino acids by either aerobic or anaerobic microorganisms, but it is not generally considered as a major source of sulphide in the anoxic system. Jorgensen (10), for example, claimed that only 3% of the total sulphide produced in the sediments of a fjord was derived from organic sources of sulphur. Nedwell and Floodgate (14), however, suggested from studies of an intertidal mud flat that the sulphate-reducing bacteria were inhibited to a larger extent than other heterotrophic bacteria at temperatures below 10°C and hence at these temperatures organic sulphur became a major source of sulphide. Jorgensen (10) could, however, find no difference between the Q_{10} of the sulphate-reducers and other bacteria in this temperature range.

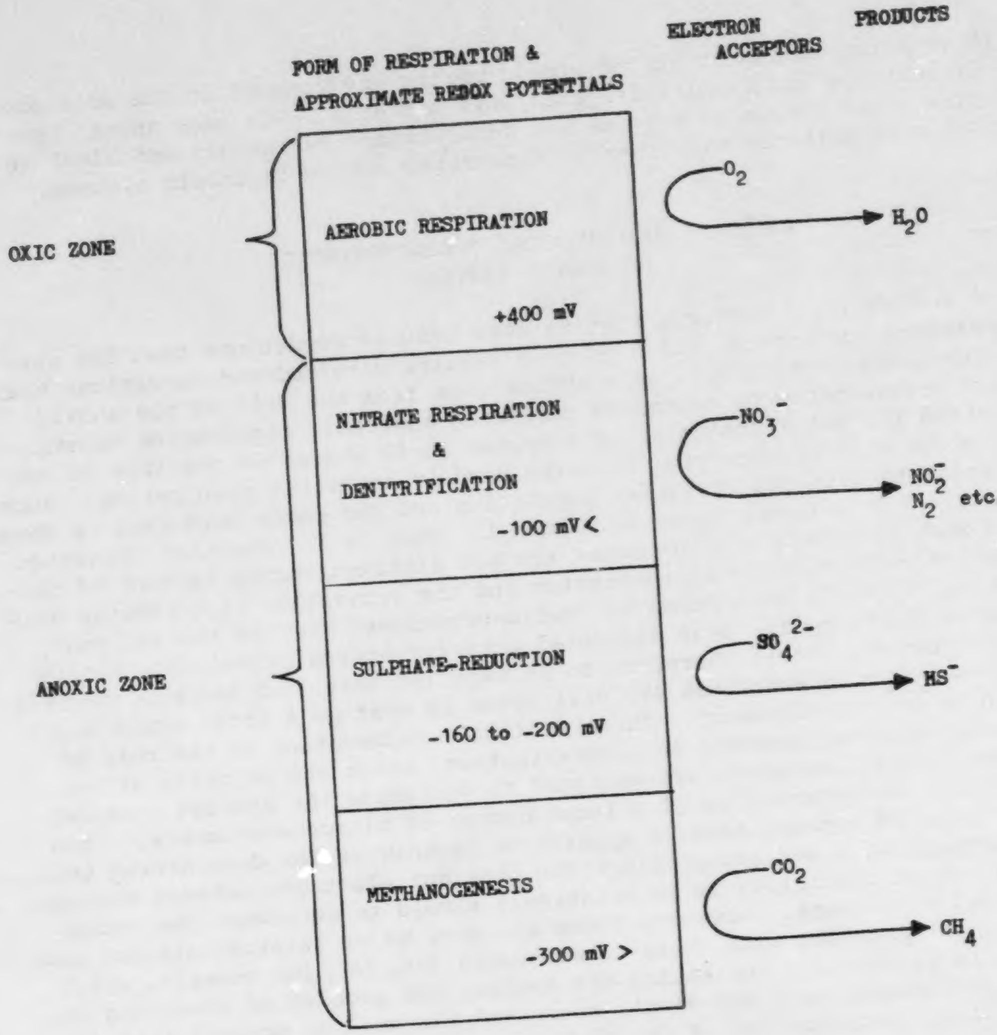


Figure 1. The relationship between anaerobic respiration, redox potential and the depth of sediment.

At the pH of most marine sediments the sulphide is present as H_2S or HS^-. The sulphide will react with any available heavy metal in forming ferrous sulphides (7). Jorgensen (10) found that 10% of the sulphide produced in a sediment was precipitated by metal ions, the rest being oxidized. Sulphide reacts rapidly with oxygen, the half life of sulphide in natural sea water being usually in the order of 10 min to one hour (1). The release of sulphide from sediments into the water column can therefore cause considerable deoxygenation (18) which in turn can cause the death of aquatic plants and animals. The release of sulphide will be enhanced by changes in factors such as pH, temperature, pressure and animal activity. Sulphide is a highly toxic compound and its presence even at very low concentrations can have lethal or marked sublethal effects on an animal or plant community (20). Sulphide is also used by many of the anaerobic photosynthetic bacteria and as a source of energy by the aerobic chemolithotrophic sulphur-oxidizing bacteria.

DEVELOPMENT AND MAINTENANCE OF ANOXIA

Diffusion of oxygen from the water column into the sediment is a relatively slow process, however, the activities of the burrowing animals in the sediment are believed to be an important factor in supplying oxygen to the sediment. These animals are not evenly distributed through the sediment, since this depends on a number of biological and physical factors (22, 26). The addition of industrial or domestic waste to sediment can cause considerable changes in the composition and activity of the burrowing animal community and hence, indirectly affect the activity of anaerobic bacteria. Such changes can be caused by the formation of a smothering blanket of particulate matter, the presence of toxic compounds or simply by increased aerobic microbial activity which causes deoxygenation.

Most of the anaerobic bacteria are inhibited by even low concentrations of oxygen. The activity of the facultative anaerobes will assist in the maintenance of an anoxic environment; in addition, the sulphide produced by the anaerobic sulphate-reducing bacteria will purge oxygen from the system. The other products of anaerobic respiration, i.e., nitrogen and methane, do not react with oxygen, hence oxygen uptake measurements of a sediment give a poor indication of anaerobic microbial activity.

FOOD WEBS

One of the major features of microbial activity in anoxic systems, whether in the marine or other environments, is the development of complex "food webs." These webs arise because, as stated earlier, fermentation is an inefficient process, producing incompletely oxidized products. The sulphate-reducing and methanogenic bacteria can utilize only a very limited range of compounds as a source of carbon and energy; these compounds are the products of fermentative microorganisms. Degradation of organic compounds in the anoxic environment therefore occurs in a number of metabolic steps involving distinct physiological and biochemical groups of microorganisms which result in denitrification, sulphate-reduction or methanogenesis (Fig. 2). There is little information on the function and activity of the "food webs" in marine systems. More is known about the activities of anaerobic bacteria in other anoxic environments, particularly the rumen; care must be exercised in transposing results from one environment to another but we can learn from the experiences of workers in these fields, for example, the need to use rigorous anaerobic techniques to isolate and grow obligate anaerobes. A major problem in studying the anaerobic "food web" is that some of the necessary experiments require pure cultures; the difficulty in isolating and maintaining pure cultures of, for example, marine, obligate anaerobic, cellulolytic bacteria can be seen by the paucity of these bacteria in culture collections. While a large number of organic compounds can be fermented by anaerobic bacteria, certain groups of organic compounds do appear to be recalcitrant, e.g., the alkanes and many of the aromatics. Aerobic degradation of aromatic compounds often involves an oxygen-dependent step, though there are a few alternative mechanisms which can be used by anaerobic microorganisms (4, 13). The fermentation products are then used by bacteria relying on anaerobic respiration which involves some interesting, and as yet largely unexplored, microbial interactions. Evidence from other anoxic environments would suggest that anaerobic mixed cultures exhibit some characteristics which are important from the ecological viewpoint. Mixed cultures can ferment a wider range of compounds than the component pure cultures, for example, propionate and benzoate can only be degraded anaerobically by mixed methanogenic cultures. In mixed cultures more growth and a greater degree of substrate utilization is usually possible than in pure cultures, also the fermentation products will differ both qualitatively and quantitatively. For example, in pure culture *Rumenococcus albus* ferments glucose to acetate, ethanol, CO_2 and hydrogen, but in a mixed culture with *Vibrio succinogens*, ethanol is no longer produced while the amount of acetate produced increases. Mixed cultures, in addition, permit energetically unfavorable reactions, e.g., the fermentation of ehtanol to acetate by the "S" organism of *Methanobacterium omelianskii*, to be "pulled through" by the removal of hydrogen during the metabolism of the second organism.

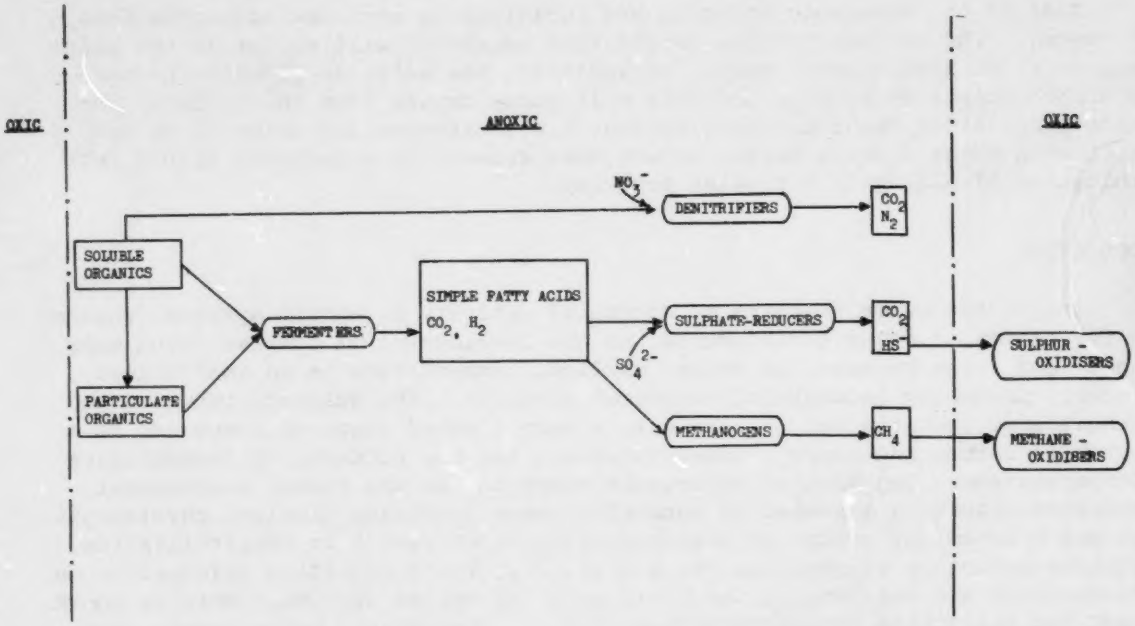
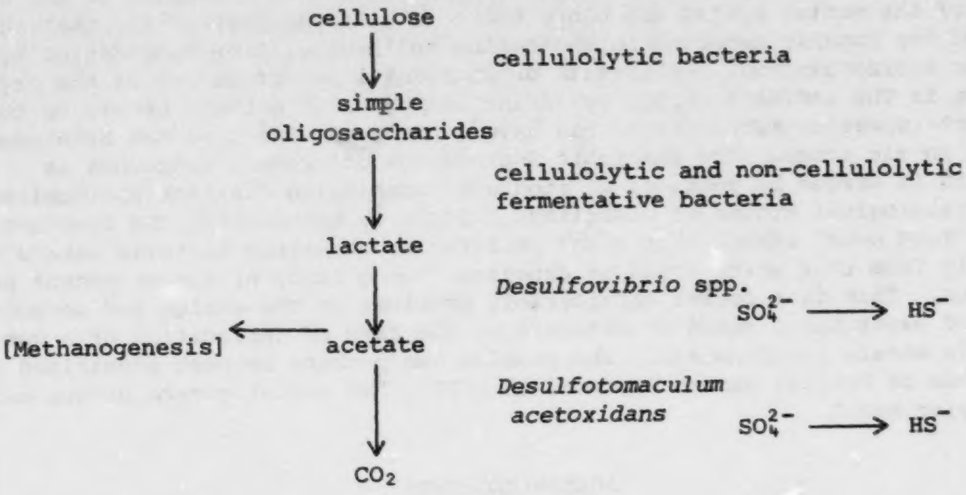


Figure 2. The basis for the formation of anaerobic food webs.

The relationship between the sulphate-reducing and methanogenic bacteria is still not certain. For example, the sulphate-reducers can produce acetate and sulphide, the former can be used as an electron donor by the methanogens, but the latter compound can also be inhibitory, hence a spatial relationship develops (3). Bryant et al. (2) from studies with known mixtures of bacteria suggested that hydrogenase-forming, sulphate-reducing bacteria could be active in some methanogenic systems that are low in sulphate. In the marine sediment it is still unclear whether methanogenesis occurs only below the sulphate-reducing zone or if both processes can occur in the sulphate-reducing zone, the methane produced being used by the sulphate-reducing and other bacteria (12). It is also probable that the acetate produced by some of the sulphate-reducing bacteria is used by acetate-utilizing sulphate-reducers; the relationship between cellulolysis and sulphate-reducers is a major interest of my group. It is, for example, possible to postualte the following mechanisms for cellulose degradation, although this is a simplified diagram because the role of hydrogen and possible inhibition caused by the production of sulphide and hydrogen is omitted.


cellulose
cellulolytic bacteria
simple oligosaccharides
cellulolytic and non-cellulolytic fermentative bacteria
lactate
Desulfovibrio spp.
$SO_4^{2-} \longrightarrow HS^-$
[Methanogenesis]
acetate
Desulfotomaculum acetoxidans
$SO_4^{2-} \longrightarrow HS^-$
CO_2

The actual construction of the food web will presumably depend on a wide range of environmental factors. The complexity of the "food web" is necessary for efficient degradation to occur, but it also means that the degradation process is particularly susceptible to disruption, for example, many microorganisms are inhibited by quite low concentrations of hydrogen and sulphide, compounds which are often end-products of the degradation process. Very recently Parkes (15) has suggested some of the seasonal changes in microbial activity observed within the sediments of the polluted estuary of the Aberdeenshire Don were caused by sulphide inhibition of the bacteria. Poole et al. (18) found up to a 24% decrease in cellulose degradation in anoxic sediments with a high sulphide concentration.

Many of the products of the anaerobic degradation processes will diffuse into the oxic zones. The transport of material between the oxic and anoxic zones is considerably assisted by the activities of the sediment-ingesting animals, if they are present. Rhoads (21) calculated that, in some sediments, these animals could cause the turnover of the whole sediment annually to a depth of 5-15 cm. Many of the aerobic bacteria are able to use the products of anaerobic metabolism as a source of carbon or energy, e.g., the sulphur- and methane-oxidizing bacteria. The "food web" responsible for the anaerobic degradation of organic ompounds therefore has ramifications in the oxic environment. There is one other aspect of the interchange between oxic and anoxic systems which has yet to be fully explained, i.e., the mechanisms by which non-sporulating, obligate anaerobic bacteria, most of which are very sensitive to oxygen, survive in and are dispersed through oxic environments; presumably the answer is that some of the vegetative cells in the population are more resistant to oxygen, or that the anaerobes survive in anoxic microenvironments, possibly in association with facultative anaerobes.

CONCLUSION

It can be concluded that the anoxic environment is extensive in the sediments of the marine system and hence there is a strong possibility that the fate of any organic compound in the marine sediment will be degradation by anaerobic microorganisms. Deliberate or accidental perturbations of the oxygen balance in the sediment, e.g., by adding compounds of a toxic nature to the sediment-ingesting macro-fauna, can have a profound effect on the development of the anoxic zones. The anaerobic degradation of organic compounds is achieved in stages by means of a "food web" comprising distinct biochemical and physiological groups of organisms. Little is known about the function of these "food webs" except that mixed cultures of anaerobic bacteria behave differently from that which would be expected from a study of the component pure cultures. This fact raises considerable problems in the design and methodology of experiments aimed at determining the rate of degradation of a compound in anoxic environments. The problem can perhaps be best summarized in the words of Francis Bacon, who said in 1620: "We cannot govern nature except by obeying her."

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Bella, D. A., A. E. Ramm, and P. E. Peterson. 1972. Effects of tidal flats on estuarine water quality. J. Water Poll. Contr. Fed. 44:541-556.

2. Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabill. 1977. Growth of Desulfovibrio in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl. Environ. Microbiol. 33:1162-1169.

3. Cappenberg, T. E. 1974. Interrelationships between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh-water lake. I. Field observations. Antonie van Leeuwenhoek J. Microbiol. Serol. 40:285-296.

4. Dagley, S. 1971. Catabolism of aromatic compound by microorganisms. Adv. Microbial Physiol. 6:1-46.

5. Fenchel, T. M., and B. B. Jorgensen. 1977. Detritus food chains of aquatic ecosystems: the role of bacteria, p. 1-57. *In* M. Alexander (ed.), Advances in microbial ecology, Vol. 1. Plenum Press, New York.

6. Fenchel, T. M., and R. J. Riedl. 1970. The sulfide system: a new biotic community underneath the oxidized layer of marine sand bottoms. Mar. Biol. 7:255-268.

7. Goldhaber, M. B., and I. R. Kaplan. 1974. The sulfur cycle. *In* E. D. Goldberg (ed.), The sea, Vol. 5. John Wiley and Sons, New York.

8. Hamilton, W. A. 1978. Microbial energetics. *In* J. Lynch and N. J. Poole (eds.), Microbial ecology: a conceptual approach. Blackwell Scientific, Oxford (in press).

9. Jorgensen, B. B. 1977. Bacterial sulfate reduction within reduced microniches of oxidized marine sediments. Mar. Biol. 41:7-17.

10. Jorgensen, B. B. 1977. The sulfur cycle of a coastal marine sediment (Limfjorden, Denmark). Limnol. Oceangr. 22:814-832.

11. Kroger, A. 1976. Phosphorylative electron transport with fumarate and nitrate as terminal hydrogen acceptors. Symp. Soc. Gen. Microbiol. 26:61-93.

12. Martens, C. S., and R. A. Berner. 1977. Interstitial water chemistry of anoxic Long Island Sound sediments. I. Dissolved gases. Limnol. Oceangr. 22:10-25.

13. Morris, J. G. 1975. The physiology of obligate anaerobiosis. Adv. Microbial Physiol. 12:169-246.

14. Nedwell, D. B., and G. D. Floodgate. 1972. Temperature-induced changes in the formation of sulphide in a marine sediment. Mar. Biol. 14:18-24.

15. Parkes, R. J. 1978. The seasonal variation of bacteria within the sediments of a polluted estuary. Ph.D. Thesis, Aberdeen University.

16. Parkes, R. J., M. J. Bryder, R. M. Madden, and N. J. Poole. 1978. Techniques for investigating the role of anaerobic bacteria in estuarine sediments. *In* G. Hamilton, Methodology for biomass determinations and microbial activities in sediments. ASTM, Philadelphia (in press).

17. Payne, W. J. 1973. Reduction of nitrogenous oxides by microorganisms. Bacteriol. Rev. 37:409-452.

18. Poole, N. J., D. J. Wildish, and N. Lister. 1976. The use of microecosystem models to investigate pollution of the estuarine ecosystem by pulp mill effluent. Manuscr. Rep. Ser. Fish Res. Bd. Can. No. 1403:1-18.

19. Poole, N. J., R. J. Parkes, and D. J. Wildish. 1977. Reaction of estuarine ecosystems to effluent from pulp and paper industry. Helgolander wiss. Meeresunters. 30:47-61.

20. Poole, N. J., D. J. Wildish, and D. D. Kristmanson. 1978. The effects of the pulp and paper industry on the aquatic environment. CRC Crit. Rev. in Environ. Control 8:153-195.

21. Rhoads, D. C. 1967. Biogenic reworking of intertidal and subtidal sediments in Barnstable and Buzzards Bay, Massachusetts. J. Geol. 75:461-476.

22. Rhoads, D. C., and D. K. Young. 1970. The influence of deposit feeding organisms on sediment stability and community trophic structure. J. Mar. Res. 28:150-178.

23. Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bact. Rev. 41:100-180.

24. Whitfield, M. 1971. Ion selective electrodes for the analysis of natural waters. Australian Marine Sciences Association, Sydney.

25. Widdel, F., and N. Pfennig. 1977. A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, *Desulfotomaculum* (emend.) *acetoxidans*. Arch. Microbiol. 112:119-122.

26. Wildish, D. J. 1977. Factors controlling marine and estuarine sublittoral macrofauna. Helgolander wiss. Meeresunters. 30:445-454.

AQUATIC ENVIRONMENT AND PESTICIDE BEHAVIOR: MICROORGANISM BINDING PHENOMENA

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ABSTRACT

A microcosm apparatus is described for studying the fate, metabolism, and movement of synthetic chemicals in the environment. This microcosm is compartmentalized and consists of terrestrial and aquatic components which can be held separately under a variety of environmental conditions. Simulated rain delivered occasionally to the terrestrial portion results in soil runoff which is channeled into the aquatic component with its layer of lake bottom mud and its animal and plant inhabitants. The microcosm can be used to study the effects of rainfall and other environmental conditions on the fate, movement, and potential bioaccumulation, and interaction of one or several test compounds after their application to soils and/or crops. Data are presented relative to the behavior of insecticides in the terrestrial and aquatic components of the microcosm. Problems of unextractable or bound ^{14}C-residues in soils and in crops, the mechanism of binding and the uptake of bound residues by worms and oats are discussed. Data relative to the fate of insecticides in water after soil runoff and the effects of lake mud deposits on the metabolism of insecticides are presented.

AQUATIC ENVIRONMENT AND PESTICIDE BEHAVIOR

One of the factors contributing to water contamination by pesticides is runoff of agricultural soils previously treated with pesticides for control purposes. Thus, soil particles contaminated with insecticide residues can be transported with runoff water into lakes and rivers where they may end up as deposits on river or lake bottom mud. Experiments conducted recently in our laboratory have dealt with: problems of the metabolism and movement of ^{14}C-phorate in a flooded soil-water-plant microcosm; the effects of lake bottom mud on the above processes; and the importance of microorganisms on the fate of ^{14}C-phorate in a soil-lake mud-water microcosm.

The first series of experiments dealt with investigation of soil flooding on the fate and metabolism of ^{14}C-phorate in an agricultural loam soil, on the movement and metabolism of the insecticide in a soil-water-plant system and factors affecting these phenomena. ^{14}C-phorate residues were readily released from submerged soils into water, amounting to 45% of applied radiocarbon during the first three days following flooding. After a two-week incubation period, as much as 50% of the radiocarbon applied to the soil was recovered from the water. Phorate was much more persistent under flooded than under nonflooded conditions. It was the major compound from submerged soils where it accounted for approximately 70% of the total residues recovered. Phorate sulfoxide was the major metabolite present in the water. In nonflooded soils, phorate sulfone was the principal metabolite, while only traces of it were detected in the flooded system. However, when *Elodea* plants were introduced into the system, after 14 days phorate sulfone amounted to 30% of all benzene-extractable ^{14}C-residues recovered, phorate sulfoxide to 44% and phorate to 27%. At that time, soil, water and plants contained 32%, 39% and 17%, respectively, of the applied radiocarbon. While more lipid-soluble volatile metabolites were recovered from nonflooded soils, more $^{14}CO_2$ was evolved from the flooded soil. The production of $^{14}CO_2$ was a function of microbiological activity. When ^{14}C-phorate treated soil was flooded with increasing amounts of water, the amounts of radiocarbon residues in the water increased. However, ^{14}C-residues in the water decreased with increasing amounts of soil (10).

The effects of lake bottom mud on movement and metabolism of ^{14}C-phorate in a flooded soil-plant system were studied with ^{14}C-phorate-contaminated loam soils deposited on lake mud sediments. A layer of lake mud underlying insecticide-treated loam soil significantly increased the persistence of phorate and reduced the amounts of ^{14}C-phorate residues released from the sediments into the water. After a two-week incubation, 62% of the applied phorate was recovered undegraded, 13% as phorate sulfoxide; however, no phorate sulfone could be detected in the presence of lake mud. When insecticide-treated loam soil was mixed with lake mud sediments, the metabolism of phorate was further reduced and even less radiocarbon moved into the water. Varying the amount of lake mud or of insecticide-treated soil did not affect insecticide distribution in the system, nor its metabolism. Both evolution of $^{14}CO_2$ and the release of volatile lipid-soluble metabolites were depressed by underlying lake mud. Studies pertaining to the rate of metabolism and movement of ^{14}C-phorate in a soil-lake mud-water-plant system over a two-week incubation period indicated a decline in the radiocarbon content of the soil-lake mud mixture during the first three days of incubation, with a concomitant increase of ^{14}C in the water. Subsequently, the amounts of ^{14}C-compounds remained constant in the soil-lake mud, decreased in the water, and increased steadily in the *Elodea* plants, which picked up these compounds from the water. Most of the plant-associated radiocarbon could not be extracted and was bound to the plant tissue, amounting finally to 81% of all the radiocarbon recovered from *Elodea* (11).

Since under agricultural field conditions phorate is oxidized rather rapidly in soil to its sulfone and sulfoxide, the experiments were conducted to investigate the fate of these compounds in soil deposited on flooded lake mud. In particular, the importance of microorganisms in reduction of phorate sulfoxide in a soil-lake mud-water microcosm was studied. By utilizing a loam-lake mud-water microcosm, the reduction of phorate sulfoxide, an oxidation product of the insecticide phorate, was demonstrated. With flooded phorate sulfoxide-treated loam soil, only small amounts of phorate were produced. The addition of lake mud, however, dramatically increased reduction of phorate sulfoxide to phorate, which after two weeks of incubation accounted for 44% of the totally recovered residues. Exposure of *Drosophila melanogaster* Meigen to dry residues of extracts of phorate sulfoxide-treated loam soil, deposited upon lake mud under flooded conditions, indicated an increase in toxicity due to production of phorate. This reduction of phorate sulfoxide to phorate was the result of the activity of microorganisms, which in turn was dependent on the supply of organic nutrients. Thus, addition of glucose to this system further enhanced the reduction of phorate sulfoxide to phorate. Phorate sulfone, another oxidative product of phorate in aerobic agricultural soils, was not reduced to phorate sulfoxide or to phorate (9).

SOIL BINDING PHENOMENA AND RELEASE OF BOUND PESTICIDE RESIDUES

Results obtained recently (1, 2, 3, 6, 7) in our laboratory pertaining to soil-bound insecticide residues and their potential release are summarized in the following discussion.

For many years depletion curves indicating the persistence or rate of disappearance of insecticides applied to soils have been published. Typical depletion curves (Fig. 1) were obtained in 1964 by our group after applying insecticides at the same rate, the same time and by the same methods to loam soil field plots near Madison, Wisconsin (5). These depletion curves indicated that compounds like aldrin or dieldrin persist in soil considerably longer than malathion, methylparathion or parathion. Frequently the decline in detectable residues has been associated with such terms as "disappearance" or "loss" or "volatilization." However, the apparent disappearance of a pesticide from soil can be due to an inability to detect its residues by conventional procedures. One reason a chemical cannot be detected is that the compound, or its degradation products, cannot be extracted from soil, thus they are "invisible." Those residues which can be extracted long after their application are the "visible," persistent ones.

The use of radiolabeled pesticides in laboratory studies has made it possible to detect unextractable soil residues. Combustion or strong hydrolysis of extracted soils can release these unextracted or bound ^{14}C-residues. The problem is complicated, since present methods for release or liberation of these bound residues also result in the destruction of their identity. Insight into the mechanism of binding of pesticide residues to soils might shed some light on the nature of the residues and their potential release.

Utilizing ^{14}C-ring labeled parathion, the amount of unextractable or bound ^{14}C-residues in a sandy and a loam soil were determined by combustion to $^{14}CO_2$, after the soils had been extracted three times with benzene-acetone-methanol (1:1:1) (10). Depletion curves of extractable parathion residues established during a one-month incubation period (Fig. 2) were similar to those established under field conditions and resulted finally in recoveries of 30 to

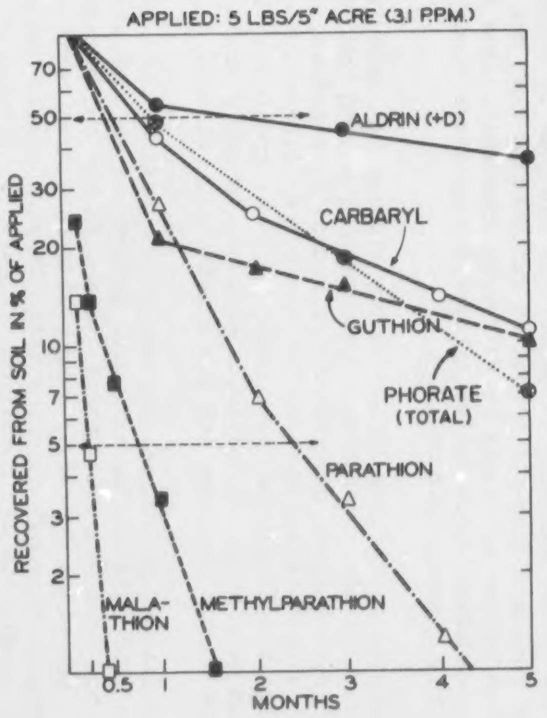

Figure 1. Depletion curves depicting extractable residues of various insecticides applied at 6 kg/ha (concentration, 3.1 ppm) to loam soils in field plots.

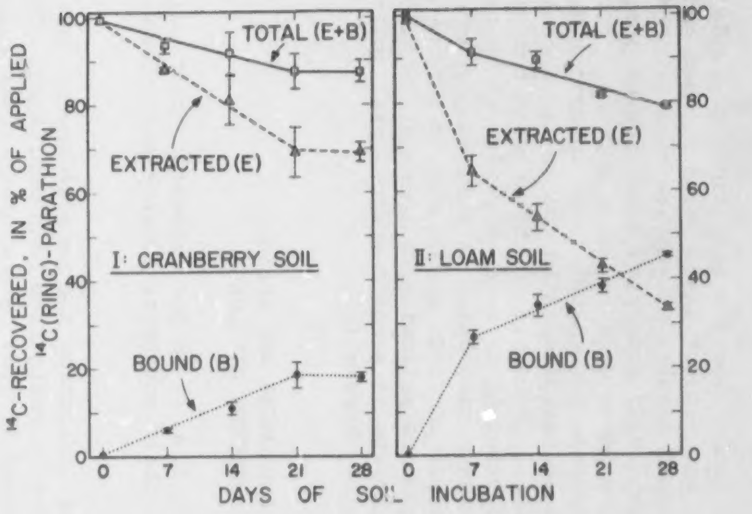

Figure 2. Binding and extractability of ^{14}C-ring-parathion in two soils during a 28-day incubation period at 27°C (applied dose, 1 ppm). The amounts of parathion as determined by GLC in the loam soil extract were nearly identical to its radiocarbon content. (I) Sandy soil from cranberry bog; (II) loam soil. Curve B, bound parathion; curve E, extracted parathion; and curve E + B, the total.

36% of the insecticide dose applied to the loam soil. With a steady decrease of extractable residues over a one-month incubation period, an increase of unextractable, bound ^{14}C-residues occurred. This resulted finally in total recoveries of extracted plus bound residues, which amounted to 80% of the applied radiocarbon. Attempts to exhaustively extract these bound ^{14}C-residues with a variety of solvents, ranging in polarity from benzene to water, failed to further release a significant amount. The rate of binding of ^{14}C-residues was highest in the loam soil and was related to the activity of soil microorganisms. In soils sterilized by gamma irradiation or by autoclaving, the binding of ^{14}C-parathion was reduced by 58 to 84%. Under anaerobic conditions, created by flooding soils with water, the rate of binding of ^{14}C-compounds doubled. The amount of bound residues decreased from 67 to 16% when soils were sterilized prior to insecticide treatment and flooding. Reinoculation of this soil with microorganisms fully reinstated the soil binding capacity. Incubation of the moist-treated soil under nitrogen also increased the formation of bound residues, while incubation at 6°C rather than 27°C inhibited binding. When these experiments were repeated using ^{14}C-ethyl rather than ^{14}C-ring labeled parathion, identical results were obtained which indicates that the bound residues apparently contain both the aryl and alkyl portions of the paration molecule. This information led us to suspect that amino-parathion, which contains both the aryl and alkyl portions of the molecule and is formed under anaerobic conditions, might be the bound residue. In addition, earlier data from our laboratory (4) indicated that while parathion residues could be extracted and detected by thin-layer chromatography in loam soil extracts after several weeks of soil incubation, amino-parathion and *p*-aminophenol could not be recovered after a one-day soil incubation. When this experiment was repeated in 1976 using radiolabeled compounds, we found that 49% of applied ^{14}C-aminoparathion could not be extracted from the soil 2 h after its application, while only 1.6% of applied parathion was bound in 2 h (3). Comparison of binding of all of the nitro and amino analogs of parathion (Fig. 3) during a brief 2-h incubation with loam soil indicated that in all instances the amino compounds were bound to a much greater extent than the nitro compounds.

The role of microorganisms in producing ^{14}C-parathion-derived bound residues and the mechanism of production of soil-bound residues was also investigated by incubating ^{14}C-ring-parathion in soil-free culture media inoculated with soil microorganisms (3). The amounts of ^{14}C-compounds in culture supernatants, that upon addition to soil became unextractable, increased up to 12 h of microbial culture incubation, when 43% of the applied radiocarbon was bound after a 2-h soil incubation period (Fig. 4). The increase in soil-bound residues correlated with a decrease in the amount of parathion in the microbial culture and a concomitant increase in appearance of the major degradation product, aminoparathion (Fig. 5).

These data indicate that the production of soil-bound residues of parathion occurs in two steps. First, microorganisms convert parathion to aminoparathion, which becomes rapidly bound to soil. These soil-bound residues are unextractable and therefore undetected in routine residue analyses.

Experiments conducted with ^{14}C-phorate (7) indicated that 26.4% of the applied residues had become soil-bound within one week of incubation. Contrary to results with ^{14}C-parathion, binding of ^{14}C-phorate residues did not increase after one week. Further investigations (6) pertaining to the

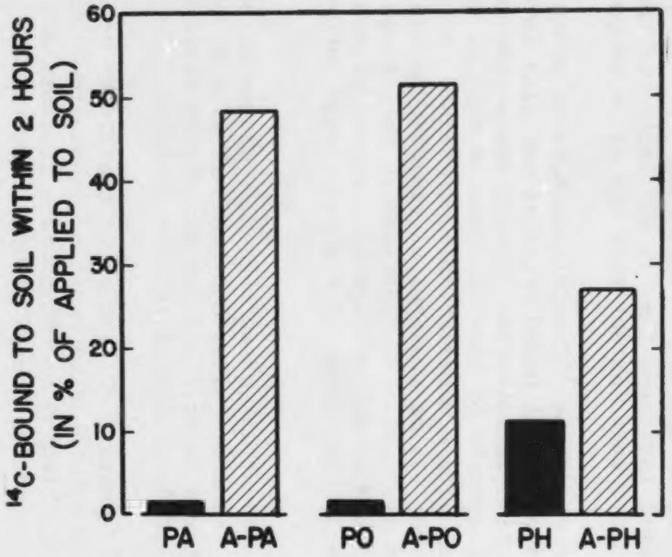

Figure 3. Amounts of ^{14}C-bound residues in soils, 2 h after soil treatment at 1 ppm with ^{14}C-parathion (PA), ^{14}C-aminoparathion (A-PA), ^{14}C-paraoxon (PO), ^{14}C-aminoparaoxon (A-PO), *p*-^{14}C-nitrophenol (PH), or *p*-^{14}C-aminophenol (A-PH).

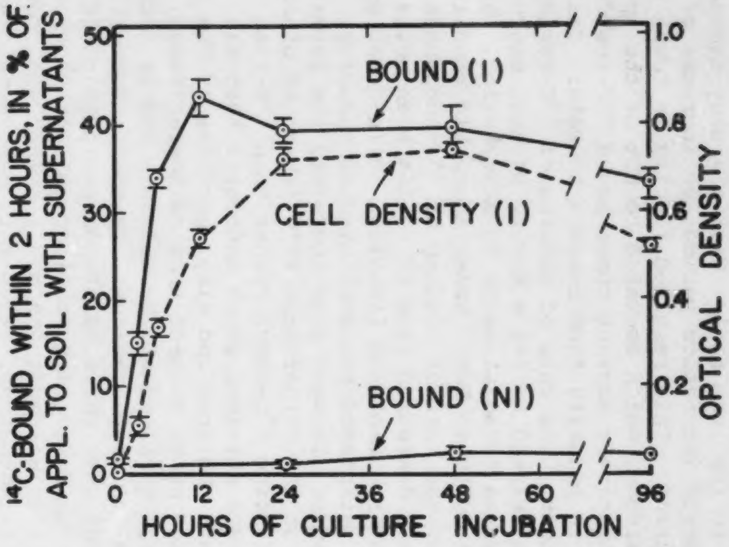

Figure 4. Binding of ^{14}C-compounds to soil within 2 h after the addition of supernatants obtained from 0- to 96-h old microbial cultures, treated with ^{14}C-parathion and inoculated (I) with soil microorganisms; NI = non-inoculated controls.

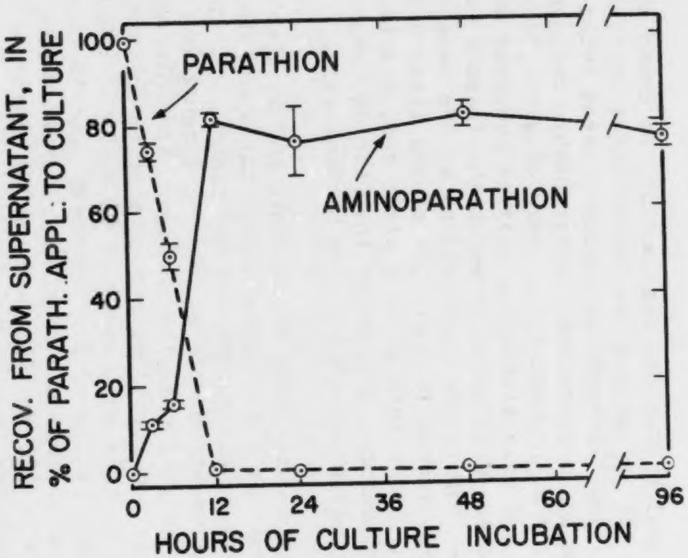

Figure 5. Amounts of parathion and aminoparathion determined by gas liquid chromatography in benzene extracts of supernatants of microbial cultures after treatment with ^{14}C-ring-parathion at 10 ppm and incubation for 0 to 96 h.

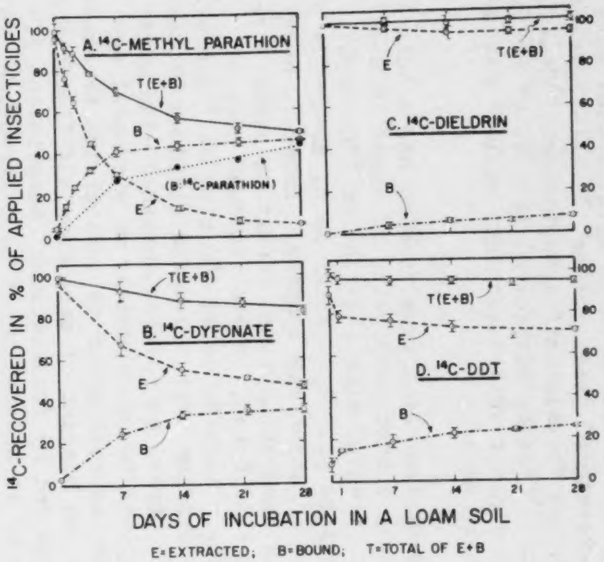

Figure 6. Binding and extractability of ^{14}C-labeled insecticides in a silt loam soil during a 28-d incubation period, after soil treatment at 1 ppm. With the exception of ^{14}C-methylparathion the amounts of extractable ^{14}C-Dyfonate (fonofos), ^{14}C-Dieldrin, and ^{14}C-DDT, as determined by gas-liquid chromatography, were similar to the amounts of extractable radiocarbon. For comparison purposes, data are inserted in A for the bound residues of ^{14}C-parathion in soil.

extractability and formation of bound ^{14}C-residues in an agricultural loam soil were conducted with the "non-persistent" insecticides ^{14}C-methylparathion and ^{14}C-fonofos (Dyfonate®) and with the "persistent" insecticides ^{14}C-dieldrin and ^{14}C-*p,p*'-DDT (Fig. 6). With ^{14}C-methylparathion, only 7% of the applied radiocarbon was extractable 28 d after soil treatment, while ^{14}C-bound residues amounted to 43% of the applied dose.

Field studies conducted in 1968-69 indicated that fonofos has a half-life in Plano silt loam soil under Wisconsin summer conditions of about 28 d (8). In the laboratory, this loam soil was treated with ^{14}C-ring or ^{14}C-ethyl-labeled fonofos and incubated for various periods. After 28 d, about 47% of the radiocarbon was extractable, most of which was fonofos. However, unextractable ^{14}C-residues increased with incubation time, resulting after 4 weeks in 35% of the applied residues being soil bound. These residues were of course not detected in the field study. Results using ^{14}C-ring or ^{14}C-ethyl labeled fonofos were very similar indicating that the bound residues probably do not involve a cleavage product. Contrary to results obtained with parathion, binding of fonofos does not appear to be dependent on microbial activity. While irradiation or autoclaving soil prior to insecticide treatment and incubation significantly reduced the binding of parathion and flooding enhanced it, fonofos binding was not reduced by irradiation. Autoclaving reduced binding somewhat, possibly due to an alteration of soil structure, and flooding slightly reduced fonofos binding. Smaller amounts of soil-bound residues had been formed with the "persistent" insecticides amounting after 28 d to only 6.5% of the applied ^{14}C-dieldrin and to 25% of the applied ^{14}C-*p,p*'-DDT, while 95% and 72%, respectively, were still recovered by organic solvent extraction. They differed from the organophosphorus compounds in their relatively low binding properties and high extractability from soils.

The question of the potential biological availability of bound insecticide residues was investigated (6) by testing the insecticidal activity of bound residues from ^{14}C-fonofos and ^{14}C-methylparathion-treated soils with fruit flies (*Drosophila*). With soils containing unextractable radiocarbon at the insecticide equivalent of 3 ppm, no mortalities were observed during a 24-h exposure period to the soil, and only slight mortalities occurred during an additional 48-h exposure. However, with soils to which the insects were exposed immediately following the insecticide application, at the same concentration as the unextractable radiocarbon (3 ppm), 50% of the flies died within 2-3 h after fonofos application, and within 18-20 h after soil treatment with methylparathion. It appears, therefore, that bound insecticide residues are not only unextractable, but they also are less active biologically.

Experiments also were conducted to study the release and availability of unextractable, soil-bound residues of ^{14}C-ring-methylparathion and the potential uptake of these ^{14}C-residues by earthworms and oat plants (1). Data from this investigation indicate that unextractable soil-bound insecticide residues are not entirely excluded from environmental interaction. After incubation of soil treated with ^{14}C-methylparathion for 14 d, and exhaustive solvent extractions, bound residues remaining in this soil amounted to 32.5% of the applied insecticide. However, after worms had lived for 2-6 weeks in this previously extracted soil containing only bound residues or after several crops of oats had grown in it, sizable amounts of ^{14}C-residues were found in the animals. Earthworms which lived in the soil for 6 weeks contained a total of 2.7% of the ^{14}C-residues which could not be extracted from these soils, while three crops of oat plants each grown for two weeks contained a total of 5.1%. The majority of previously soil-bound ^{14}C-residues taken up by earthworms (58-66%) again became bound within the animals, while most (82-95%) of the ^{14}C-residues in oat plants were extractable. Greens of oat plants contained 46-62% of the ^{14}C-residues recovered from plants. Most of the ^{14}C-residues in oat greens were benzene-soluble while most of the ^{14}C-residues in the seeds and roots were water-soluble.

Because soil-bound insecticide residues can be released from soil by these organisms, any loss in toxicity due to binding should not be regarded as permanent. Even if release of non-toxic compounds occurs, interaction with other chemicals in the environment cannot be disregarded. The release and potential biological activity of these bound residues certainly warrants further study. In view of the above finding, the expression "disappearance" and "persistence" of pesticides, so widely used during the last two decades, should be reassessed to consider the bound products.

LITERATURE CITED

1. Fuhremann, T. W., and E. P. Lichtenstein. 1978. Release of unextractable soil bound ^{14}C-methylparathion residues and their uptake by earthworms and oat plants. J. Agric. Food Chem. 26:605-610.

2. Katan, Y., T. W. Fuhremann, and E. P. Lichtenstein. 1976. Binding of ^{14}C-parathion in soil: a reassessment of pesticide persistence. Science 193:891-894.

3. Katan, Y., and E. P. Lichtenstein. 1977. Mechanism of production of soil bound residues of ^{14}C-parathion by microorganisms. J. Agric. Food Chem. 25:1404-1408.

4. Lichtenstein, E. P., and K. R. Schulz. 1964. The effects of moisture and microorganisms on the persistence and metabolism of some organophosphorus insecticides in soils, with special emphasis on parathion. J. Econ. Entomol. 57:618-627.

5. Lichtenstein, E. P. 1975. Transport mechanism in soil: metabolism and movement of insecticides from soil into water and crop plants. IUPAC Congress of Pesticide Chemistry, Helsinki, 1974. Pure Appl. Chem. 42:113-118.

6. Lichtenstein, E. P., Y. Katan, and B. N. Anderegg. 1977. Binding of "persistent" and "nonpersistent" ^{14}C insecticides in agricultural soil. J. Agric. Food Chem. 25:43-47.

7. Lichtenstein, E. P., T. T. Liang, and T. W. Fuhremann. 1978. A compartmentalized [illegible]crocosm for studying the fate of chemicals in the environment. J. Agric. Food Chem. 26:948-953.

8. Schulz, K. R., and E. P. Lichtenstein. 1971. Persistence and movement of Dyfonate in field soils. J. Econ. Entomol. 64:283-287.

9. Walter-Echols, G., and E. P. Lichtenstein. 1977. Microbial reduction of phorate sulfoxide to phorate in a soil-lake mud-water microcosm. J. Econ. Entomol. 70:505-509.

10. Walter-Echols, G., and E. P. Lichtenstein. 1978. Movement and metabolism of ^{14}C-phorate in a flooded soil system. J. Agric. Food Chem. 26:(May-June).

11. Walter-Echols, G., and E. P. Lichtenstein. 1978. Effects of lake bottom mud on the movement and metabolism of ^{14}C-phorate in a flooded soil-plant system. J. Agric. Food Chem. 26:599-604.

4

ROLE OF SURFACE MICROLAYERS

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ABSTRACT

A definition of the different surface microlayers and the distribution of "dry" and "wet" surfactants in the air/water film has been presented. Based on results from various hydrophobic samplers a thickness of 1 μm for the bacterioneuston microlayer has been established. Interest has focused on the surface lipid film not only as it may be per se of basic importance for functioning of the surface microlayer but also due to the analogous, important interface accumulation occurring in the system, namely that at the surface of gas bubbles. Some bacteria have a greater tendency to accumulate in the lipid film than others (model system studies). The number of accumulated bacteria differ with the lipid content of the film, shown in model systems and in the natural environment. Surface balance studies confirm the concept of bacterial interaction at "film-bacteria-sites" where hydrophobic interaction, surface charge of bacteria as well as of the film, and enzymic activity may be involved. Biodynamics of the surface microlayer have been dealt with considering the reported low bacterial biochemical activity, in spite of the high concentration of dissolved organic carbon in the layer.

The surface microlayers in aquatic environments are an infinitesimal part of the water body, but nevertheless are of great importance. These microlayers are dramatically different from the bulk-subsurface water, for they comprise an accumulation layer for dissolved substances, particles and microorganisms diffusing or brought by rising bubbles, convection and upwelling, from the subsurface water to the surface. Microlayers influence the exchange of gas and transport mechanisms from the water body to the atmosphere and vice versa. Furthermore, they are a sink for atmospheric fallout, and concentrate heavy metals (e.g., 13) and pollutants. The occurrence of organochlorine residues is of considerable interest. Compared to the levels of such compounds in seawater, Duce et al. (13) and Larsson et al. (22) report enrichment factors of 10^3-10^4 for PCB in samples taken from Narragansett Bay, Rhode Island,

and Jorefjorden, Sweden, respectively. The overall picture for the surface microlayers is well known. However, while our knowledge of their chemistry is gradually growing, real understanding of their biological structure and function is very far from complete.

DEFINITION OF SURFACE MICROLAYERS

First of all we have to define the term "surface microlayers." Most workers (e.g., 15, 17, 18, 22) have considered lipids to be the major constituents of the uppermost surface microlayer. The amphiphilic molecules have a preferred orientation with respect to the water surface, with the long-chained hydrophobic parts extending into the air which enable them to form a strictly ordered lipid film. This film consists mainly of free fatty acids in the C_{12}-C_{22} range, alcohols, and glycerides, of which triglycerides dominate. There are characteristic deviations in the fatty acid patterns of the film compared to that of the subsurface water. The relative amount of saturated acids is higher in the surface film compared to the subsurface water, probably because of oxidation at the air/lipid interface. The amount of glycerides relative to fatty acids is always higher in the surface multifilms than in the subsurface water. The lipid film of so-called "dry surfactants" is a real surface microlayer, based on the length of fatty acids 10-20 Å thick; if multilayered, approximately 100 Å thick.

Baier et al. (4), however, consider all waters, except heavily polluted ones, to be coated with films of polysaccharide-protein complexes ranging in thickness from 100 to 300 Å. Their statements are based on germanium-slide collected material, analyzed by a "nondestructive, direct analytical technique," multiple attenuated infrared reflectance, MAIR (3, 4). These "wet surfactants" can form a more irregular, submerged stratum, and are essentially hydrophilic but stick to the surface water by virtue of their few hydrophobic chains. As pointed out by Wangersky (34), it remains to be seen whether the infrared absorption technique overestimates the contribution of proteinaceous material.

These two strata taken together have been called the *surface microlayer* by Sieburth et al. (32), who quote a thickness of 0.1 µm. As seen in Table 1, the various sampling devices used collected surface microlayers from 250 to 4 µm thick due to the amount of water withdrawn. Most authors use the term surface microlayer in a way that makes its thickness dependent on the particular collection method employed. Sieburth et al. (32), however, designate their screen-collected layer (150 µm) simply as "screen."

In view of our special interest in the sampling of the lipid film, the efficiencies of pronounced hydrophobic samplers with limited water withdrawal were compared (Table 2). These were tested in model systems with various lipids applied at the air/water interface in different amounts, calculated to give gaseous or condensed monolayers, or a decalayer. Good yields were obtained with monolayers, but the yields of gaseous films, less lipid material than one monolayer, are significantly lower. When using the different samplers in natural environment or in a model system, the hydrophobic Nuclepore membrane gave the highest enrichment factor (E) for bacteria in all comparable experiments, varying over a range of 10^2-10^4. The factor E was defined as the number of bacteria/ml of the collected layer relative to that of the subsurface water. Parker and Hatcher (29) suggested a nonlinear change in algal population densities with surface microlayer depth, some sort of microstrata. Here the operationally dependent dimension, 1 µm, seems to coincide fairly well with a biological stratum, the bacterioneuston layer, yet another surface microlayer.

TABLE 1. VARIOUS DEVICES FOR SAMPLING OF SURFACE MICROLAYERS

Type of sampler	Sample thickness (µm)	Source	References
Screen	250-150	Seawater	(14, 31, 32)
Rotating hydrophilic drum	60	Seawater	(17)
Bubble bursting	10	Seawater model system	(6)
Hydrophilic Nuclepore membrane	40-4	Seawater estuarine	(1, 10)
Teflon plate	36-5	Seawater model system	(20, 26, 28)

TABLE 2. LIPID COLLECTING ABILITY AND WATER WITHDRAWAL OF NUCLEPORE MEMBRANES AND TEFLON SAMPLERS USED IN MODEL SYSTEMS. Data obtained from S. Kjelleberg, Th. A. Stenström, and G. Odham (to be published).

Sampler	Amount of water withdrawn[a] (µl·dm^{-2})	Calculated sample thickness (µm)	Percent recovery of lipid films: Oleic acid, Monolayer	Oleic acid, Decalayer	Olive oil (mainly triglycerides), Monolayer	Olive oil (mainly triglycerides), Trilayer	Tryglycerides of isotridecanoic acid (gaseous film)
Hydrophobic Nuclepore membrane	8±5	0.8	n.d.[b]	93	59	94	45
Hydrophilic Nuclepore membrane	294±62	29	n.d.	n.d.	n.d.	n.d.	n.d.
Teflon sheet	65±29	7	80	99	72	62	45
Teflon plate	356±90	36	99	83	83	124	52

[a] Based on 20 weighings. [b] n.d. = not determined.

Thus, the term surface microlayer has been involved in the assignment of a series of strata. To obtain uniformity, it is suggested that the term surface microlayer be used only with the 0.1 µm layer of dry and wet surfactants, and otherwise with additions of explanatory pre- or postfixes. In this manner, the strata sequence from the uppermost layer, very similar to the Top-millimeter-range of MacIntyre (24), would be: lipid film (10-100 Å), surface microlayer (0.1 µm), bacterioneuston microlayer (1 µm). As a further example, screen microlayer (e.g., 150 µm, see above) would be used as the term is operationally defined. Surface microlayers would represent the layers altogether.

ARE LIPID FILMS OF ANY SPECIAL INTEREST?

Our interest has been focused on the lipid film not only as it may be per se of fundamental importance for the function of the surface microlayers, but also due to the analogous, important interface accumulation occurring in the system, namely, that at the surface of gas bubbles. The latter are produced in different ways (e.g., by living organisms, sea turbulence, etc.) in amounts sufficient to cover 3 or 4% of the sea surface at any moment (24). During their slow rise, lipids, other organic molecules, particulate matter, and microorganisms adsorb at the surface of the bubbles, finally causing the surface microlayers to be enriched in organic matter, dead or alive. At the water surface, the bubbles eventually burst at an assumed rate of 10^8 sec^{-1}. When the surface-free energy of the bubble is transformed to the water beam as kinetic energy, the aerosol droplets formed are ejected into the air in millisecond reactions. This formation of droplets has been described as a surface microtome (23), as the ejected droplets carry away material only from the bubble surface and the top micrometers of the water surface. The rising bubbles act to link the subsurface, the surface microlayers and the atmosphere. They are of a special importance as conveyors into the atmosphere of persistent substances like PCB, which withstand most degradation processes except photodegradation.

MICROBIAL ACCUMULATION AT THE SURFACE MICROLAYERS

The microflora of the surface microlayers have been reported to be different from, and in greater concentrations than in the subsurface water (5, 6, 29). Based on these observations, a marine model system study was undertaken to find out whether some bacteria have a greater tendency to accumulate in the surface film than others, and whether the number of accumulated bacteria differ with the chemical composition or lipid content of the lipid film (26).

The model systems comprised an assay tray with a 2.5% NaCl solution as subsurface water and were inoculated with a defined number of bacteria and covered with an applied lipid surface film, mono- or decalayered. The bacteria were retrieved by the Teflon plate sampling procedure and enumerated by plate count methodology. The enrichment factor (E) for the bacteria was defined, as above, as the number of bacteria/ml in the Teflon-plate collected layer relative to that in the subsurface water. E varied over a range from 7-100 (Table 3) in a system with monolayered lipid film of oleic acid ($C_{18:1}$) and four different marine bacteria used as test organisms (*Aeromonas dourgesi*, *Pseudomonas fluorescens*, *P. halocrenaea*, and *Serratia marinorubra*). This terminology does not conform to that of Bergey's 8th Edition. *S. marinorubra* showed an E of 100 or approximately 100, about 10 times higher than values for the three other microorganisms. Still lower values were obtained for two gram-positive bacteria tested. E was of the same magnitude whether the film was formed solely of oleic acid or of an oleic acid or of an oleic-palmitic acids-triolein mixture, both monolayered. For a decalyer of oleic acid, E was approximately twice that obtained in a monolayer. Field studies also showed a positive correlation between the number of bacteria in the surface layer and the amount of lipids simultaneously collected (Table 4).

TABLE 3. ENRICHMENT FACTOR (E) FOR SOME MARINE BACTERIA IN MODEL SYSTEMS WITH MONOLAYERED LIPID FILM OF OLEIC ACID

Test organisms	Number of bacteria x 10^6 per ml in samples from		Enrichment factor (E)
	Surface film	Subsurface water	
Aeromonas dourgesi	32	3.1	10
Pseudomonas fluorescens	3.3	0.54	6
	4.0	0.69	6
	49	5.8	8
	42	5.9	7
Pseudomonas halocrenaea	22	3.2	7
	75	3.6	21
	70	8.9	8
	130	9.4	14
Serratia marinorubra	170	1.9	90
	190	1.9	100
	370	3.8	97
	410	4.3	95

TABLE 4. ENRICHMENT FACTOR (E) FOR BACTERIA AND TOTAL AMOUNT OF FATTY ACIDS IN SAMPLES FROM SURFACE (10 TEFLON PLATE SAMPLINGS) AND SUBSURFACE WATER IN A NON-POLLUTED AREA (GULLMARSFJORDEN) AT THREE DIFFERENT TIMES OF THE YEAR

Date	Number of bacteria* per ml in samples from		E	Total amount (µg) of fatty acids in samples from	
	Surface	Subsurface		Surface (1 ml)	Subsurface (1000 ml)
Aug 23 1974	12,600±870	165±26	76	24	22
Oct 24 1974	2,200±360	200±28	11	11	6.6
Nov 28 1974	5,200±560	340±37	15	13	1.9

*Average value from generally six samplings.

The E values of *S. marinorubra* decreased with an increase in the number of cells in the subsurface water, consisting solely of *Serratia* cells (Fig. 1). This might be due to a decrease in accessible "film-bacteria-sites" until a point of saturation, after which further change in the absolute number of accumulated bacteria in the film occurred only within experimental error. Even when using an almost constant number of *Serratia* cells in the bulk, in addition to increasing numbers of *Aeromonas* cells, the E values for *Serratia* decreased (Table 5). Thus, in spite of the low accumulation tendency for *Aeromonas* cells, they seem to compete for "film-sites," available binding groups in the film, or a minimum of free space between bacteria, may be necessary to eliminate the effect of electrostatic repulsion.

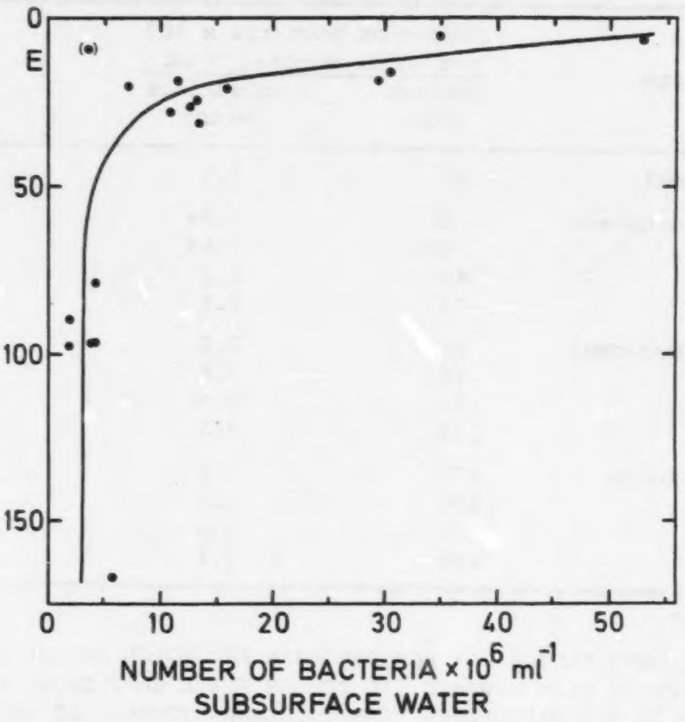

Figure 1. Enrichment factor (E) of *Serratia marinorubra* in model systems with monolayered lipid film of oleic acid at different cell densities in the subsurface water (26).

In bubble bursting experiments (6), where relatively high numbers of bacteria were used, the number of bacteria transferred into the air were also relatively constant irrespective of the number of bacteria in the solution from which they were ejected. Even here a competition for film-sites, namely around the bubble, can be assumed. The low E values obtained in Öresund, a polluted area with high numbers of subsurface bacteria, 10^5-10^8 ml^{-1} (B. Stehn, personal communication) may be explained in a similar way.

TABLE 5. ENRICHMENT FACTOR (E) FOR *Serratia marinorubra* AND *Aeromonas dourgesi* WHEN BOTH BACTERIA HAVE BEEN ADDED TO THE SUBSURFACE WATER, THE FORMER AT ALMOST THE SAME CELL DENSITY, THE LATTER AT INCREASING DENSITY

Test organisms	Number of bacteria x 10^6 per ml in samples from: Surface film, Each species	Surface film, Total	Subsurface water, Each species	Subsurface water, Total	Enrichment factor (E), Each species	Enrichment factor (E), Total
S. marinorubra	205	207	1.9	2.23	108	114
A. dourgesi	2		0.33		6.1	
S. marinorubra	127	149	2.3	8.8	55	58
A. dourgesi	22		6.5		3.4	
S. marinorubra	56	96	2.6	13.6	22	26
A. dourgesi	40		11		3.6	

MICROBIAL INTERACTION AT THE LIPID FILM-SURFACE BALANCE STUDIES

The idea of bacterial interaction at sites in or just beneath the surface film is supported in surface balance studies with various monolayered films (oleic acid, a mixture of oleic acid, palmitic acid and triolein, triolein alone, or dipalmitoyl-phosphatidylcholine) when their behavior with and without bacteria present is compared (20). During compression, the film changes from a gaseous state, with a large area per molecule, to a condensed monolayer where the molecules are tightly packed. The adhesion of bacteria (or molecules) is indicated by an increase in the surface pressure at a given area per molecule. Dissolution of the film, e.g., as a result of enzymatic activity, decreases the film pressure. As could be expected with regard to the E values obtained in similar model systems, *S. marinorubra* (Fig. 2) again showed the strongest interaction and greatest strengthening of the film, except for the cell wall-free *Acholeplasma laidlawii* (Fig. 3) which has its lipid bilayered plasma membrane accessible. From enlarged and more sophisticated surface balance sutdies (Kjelleberg and Stenström, to be published) it can be assumed that various factors besides hydrophobic interaction are involved in the interaction, e.g., surface charge, both of the monolayered surface film and of the bacteria, and enzymatic activity.

BIODYNAMICS OF THE SURFACE MICROLAYERS

The above forces, evidently different for different bacteria, may contribute to the maintenance of the bacteria in the microlayers. But how do they get there and do they reproduce in the microlayers? Aerotaxis and possibly chemotaxis may play a role in the accumulation. These factors certainly

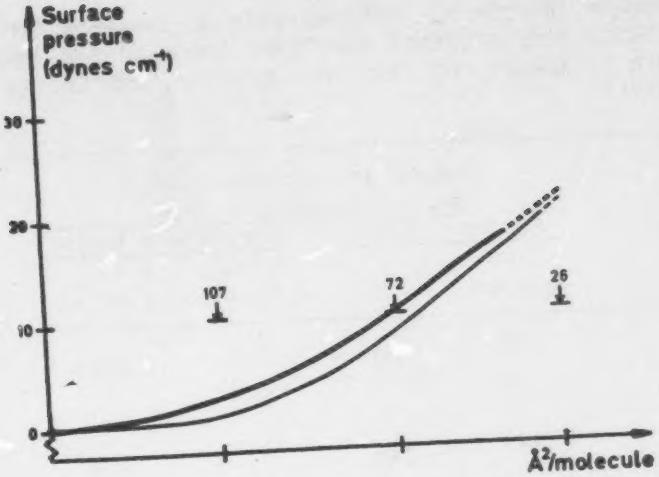

Figure 2. Surface pressure, dynes per centimeter, as a function of molecular area for a monolayer of oleic acid on saline solution with (▬▬) and without (——) cells of *Serratia marinorubra*. Broken lines indicate viscous film. The calculated molecular area (Å^2) is given at arrowed horizontal lines (21).

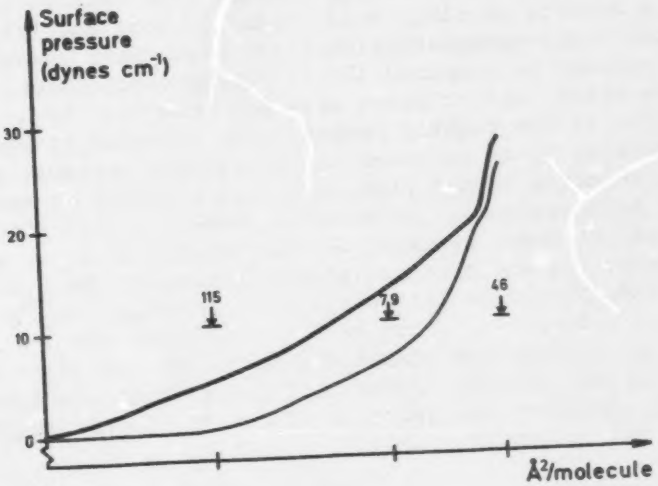

Figure 3. Surface pressures, dynes per centimeter, as a function of molecular area for a monolayer of dipalmitoyl-phosphatidylcholine on saline solution with and without cells of *Acholeplasma laidlawii A*. Same symbols as in Figure 2 (21).

are more important in model systems where forces like bubble rising, water movements, and particle transport, which are decisive in nature, do not occur. In this respect and in many others, the model systems, even in more complicated form than our simple ones, lose the multidimension of a natural ecosystem. They do, however, offer great advantages in that environmental factors may be kept constant and individual variables can be studied under controlled conditions. The results obtained can only increase our understanding of structure and function of the surface microlayers.

Natural seawater is an extremely dilute nutrient solution, too dilute for growth of most marine bacteria. Since accumulation of organic matter occurs at all interfaces, accumulation at the surface microlayers sufficient to sustain bacterial reproduction has been assumed. Aggregates in the surface microlayers, as well as subsurface aggregates, can function in the interchange of metabolites and reduction of exoenzyme escape (19). Dissolved organic carbon, in concentrations of $\leq$1.3 mg carbon liter^{-1} in the subsurface water has been estimated compared to concentration of 1427 mg liter^{-1} (2.9 g or organic matter liter^{-1}) as a mean in the 0.1 μm surface microlayer of the North Atlantic (32), which corresponds to the amount of nutrients present in media generally used to grow marine bacteria. Thus, lack of nutrients may not limit reproduction. However, potentially detrimental factors such as intense solar radiation, temperature and salinity flux, high redox potential, and the presence of toxic organic substances (32) and heavy metals may present special demands for survival and reproduction of the microorganisms. Data now being accumulated on their biochemical activity point to a low bacterial activity. Dietz et al. (12) in their comprehensive data on material from the Vancouver area note that ATP levels, heterotrophic potential, and heterotrophic activity were lower for bacterioneuston (from 70-80 μm depth) than for bacterioplankton, and per CFU, heterotrophic potential and heterotrophic activity represented only 33 and 10% respectively, of that for bacterioplankton. Kjelleberg and Håkansson (20) found a very clear trend indicating a higher number of biochemically active bacteria (lipolytic, proteolytic, and amylolytic activity being tested) in the bulk than in the surface microlayers (20 μm depth) at the Swedish West Coast. Marumo et al. (25), finally, consider the bacteria to accumulate in the surface microlayers by physical forces rather than by reproduction since an extremely small viable count relative to the total was found; most cells in "the film were dead or nearly dead." For easy testing of metabolic state in bacteria, it is necessary to find a fluorogenic, real true vital strain, comparable to the fluorescein diacetate successfully used for recognizing metabolically active fungal cells (33).

From our work on sediment bacteria, mainly lipolytic, in the deep Norwegian Sea (27), it occurred to us that conventional substrates do not offer bacteria, dependent on interfacial enzymes, suitable conditions to display their biochemical activity. The same may be valid also for bacterioneuston bacteria. There is a special interest concomitant with findings in the surface microlayers, but not in the subsurface water, of bacteria with degrading potential for compounds which, under prevailing conditions, may be assumed to accumulate in the surface microlayers (e.g., NTA-degrading bacteria [7]).

The bacterioneuston is poorly characterized, for most often we are informed that marine bacterioneuston is formed by "small, gram negative rods."

This is of minimum value since about 95% of marine bacteria are small, gram negative rods. The information "some pigmented" is just as enlightening. Carty and Colwell (9), however, classified bacterial isolates from the air/sea interface obtained during a Pacific Ocean expedition. They found *Pseudomonas, Vibrio, Aeromonas,* and *Spirillum* species predominent. The same marine species were also found in the air above the sea surface otherwise dominated by *Bacillus* spp. Even if rare and undescribed species can be expected in this hostile environment, it seems unimportant to be able to separate the bacterioneuston into genera and species. Of more importance is knowledge of its biochemical activity and means to determine in-situ activity and reproduction rate.

When collecting microalgae in surface microlayers by drum sampler (60 μm thick) in freshwater, Parker and Hatcher (29) found the seasonal distribution of algal genera to be random, with no genus consistently inhabiting either the surface microlayers or the subsurface water from 10 cm depth. However, for individual sampling dates, each genus generally was primarily concentrated either in the surface or in the subsurface water. Differences in the organic and inorganic chemistry of surface microlayers, relative to subsurface water, may contribute to differences in growth rates and resulting population size. Knowing the ease with which bacterial enrichment cultures can be obtained, such differences surely appear also in the bacterioneuston when growth and reproduction on the whole can occur.

The 1 μm surface microlayer is a heterotrophic microbial community. Besides bacteria, yeasts and "moulds" have been found (10; Kjelleberg and Stenström, to be published), the latter most probably deriving from an atmospheric spore fallout. There are also some heterotrophic microflagellates and all probably serve as food for grazing phagotrophic protists and feed the special blue-pigmented copepods floating on the surface (11). Generally we consider bacteria as decomposers and may overlook their role as food for various consumers. Sorption to, or uptake by, bacteria of various persistent compounds may serve as a means of introducing these toxic compounds into aquatic food chains (16, 30), probably of importance in accumulation layers like the surface microlayers.

Already the size of phytoplanktons surely exclude them from the bacterioneuston layer. Several investigations, however, show the presence of phytoplankton in screen microlayer samples (150-250 μm). These investigations have been based on microscopical work (e.g., 8) combined with analysis for chlorophyll *a* or phaeopigments (32). Estimations of in-situ activity, for example, expressed as C assimilated per mg photopigment and hour, however, belong to the future which we hope will add to our still marginal knowledge of the biological significance of the surface microlayers.

LITERATURE CITED

1. Ahearn, D. G., S. A. Crow, and W. L. Cook. 1977. Microbial interactions with pesticides in estuarine surface slicks. U.S. Environmental Protection Agency Rep. Ecological Res. Series 600-3.77.050.

2. Baier, R. E. 1972. Organic films on natural waters: their retrieval, identification, and modes of elimination. J. Geophys. Res. 77:5062-5075.

3. Baier, R. E. 1975. Applied chemistry at protein interfaces. Adv. in Chemistry Series 145, 387 pp.

4. Baier, R. E., D. W. Goupil, S. Perlmutter, and R. King. 1974. Dominant chemical composition of sea-surface films, natural slicks, and foams. J. Recherches Atmos. 8:571-600.

5. Bezdek, H. F., and A. F. Carlucci. 1972. Surface concentration of marine bacteria. Limnol. Oceanogr. 17:566-569.

6. Blanchard, D. C., and L. Syzdek. 1970. Mechanism for the water-to-air transfer and concentration of bacteria. Science 170:626-628.

7. Bourquin, A. W., and V. A. Przybyszewski. 1977. Distribution of bacteria with nitrilotriacetate-degrading potential in an estuarine environment. Appl. Envir. Microbiol. 34:411-418.

8. Brockmann, U. H., G. Kattner, G. Hentzschel, K. Wandschneider, H. D. Junge, and H. Hühnerfuss. 1976. Natürliche Oberflächenfilme im Seegebiet vor Sylt. Mar. Biol. 36:135-146.

9. Carty, C., and R. R. Co[illegible] 1975. A microbiological study of air and surface water microlayers in the open ocean. J. Wash. Acad. Sci. 65:148-153.

10. Crow, S. A., D. G. Ahearn, W. L. Cook, and A. W. Bourquin. 1975. Densities of bacteria and fungi in coastal surface films as determined by membrane-absorption procedure. Limnol. Oceanogr. 10:602-605.

11. David, P. M. 1965. The surface fauna of the ocean. Endeavour 24:95-100.

12. Dietz, A. S., L. J. Albright, and T. Tuominen. 1976. Heterotrophic activities of bacterioneuston and bacterioplankton. Can. J. Microbiol. 22:1699-1709.

13. Duce, R. A., J. G. Quinn, C. E. Olney, S. R. Piotrowicz, B. J. Ray, and T. L. Wade. 1972. Enrichment of heavy metals and organic compounds in the surface microlayer of Narragansett Bay, Rhode Island. Science 176:161-163.

14. Garrett, W. D. 1965. Collection of slickforming material from the sea surface. Limnol. Oceanogr. 10:602-605.

15. Garrett, W. D. 1967. The organic chemical composition of the ocean surface. Deep Sea Res. 14:221-227.

16. Grimes, D. J., and S. M. Morrison. 1975. Bacterial bioconcentration of chlorinated insecticides from aqueous systems. Microb. Ecol. 2:43-59.

17. Harvey, G. W. 1966. Microlayer collection from the sea surface: a new method and initial results. Limnol. Oceanogr. 11:608-613.

18. Jarvis, N. J. 1967. Absorption of surface-active material at the sea-air interface. Limnol. Oceanogr. 12:213-221.

19. Jones, G. E., and H. W. Jannasch. 1959. Aggregates of bacteria in sea water as determined by treatment with surface active agents. Limnol. Oceanogr. 4:269-276.

20. Kjelleberg, S., and N. Håkansson. 1977. Distribution of lipolytic, proteolytic, and amylolytic marine bacteria between the lipid film and the subsurface water. Mar. Biol. 39:103-109.

21. Kjelleberg, S., B. Norkrans, H. Löfgren, and K. Larsson. 1976. Surface balance study of the interaction between microorganisms and lipid monolayer at the air/water interface. Appl. Environ. Microbiol. 31:609-611.

22. Larsson, K., G. Odham, and A. Södergren. 1974. On lipid films on the sea. I. A simple method for sampling and studies of composition. Mar. Chem. 2:49-57.

23. MacIntyre, F. 1968. Bubbles: a boundary-layer "microtome" for micron-thick samples of a liquid surface. J. Phys. Chem. 72:589-592.

24. MacIntyre, F. 1974. The top millimeter of the ocean. Sci. Amer. 230:62-77.

25. Marumo, R., N. Tuga, and T. Nakai. 1971. Neustonic bacteria and phytoplankton in surface microlayers of the equatorial waters. Bull. Plankton Soc. Japan 18:36-41.

26. Norkrans, B., and F. Sörensson. 1977. On the marine lipid surface microlayer--bacterial accumulation in model systems. Botanica Marina 20:473-478.

27. Norkrans, B., and B. Stehn. 1978. Sediment bacteria in the deep Norwegian Sea. Mar. Biol. 47:201-209.

28. Odham, G., B. Norén, B. Norkrans, A. Södergren, and H. Löfgren. 1978. Biological and chemical aspects of the aquatic lipid surface microlayer. Progr. Chem. Fats Lipids 16:31-44.

29. Parker, B. C., and R. F. Hatcher. 1974. Enrichment of surface freshwater microlayers with algae. J. Phycol. 10:185-189.

30. Paris, D. F., and D. L. Lewis. 1976. Accumulation of methoxychlor by microorganisms isolated from aqueous systems. Bull. Environ. Contam. Toxicol. 15:24-32.

31. Sieburth, J. McN. 1965. Bacteriological samplers for air-water and water-sediment interfaces, p. 1064-1068. *In* Ocean science and ocean engineering, Trans. Joint Conf. MTS and ASLO, Washington, D.C.

32. Sieburth, J. McN., P.-J. Willis, K. M. Johnson, C. M. Burney, D. M. Lavoie, K. R. Hinga, D. A. Caron, F. W. French III, P. W. Johnson, and P. C. Davis. 1976. Dissolved organic matter and heterotrophic microneuston in the surface microlayers of the North Atlantic. Science 194:1415-1418.

33. Söderström, B. E. 1977. Vital staining of fungi in pure cultures and in soil with fluorescein diacetate. Soil Biol. Biochem. 9:59-63.

34. Wangersky, P. J. 1976. The surface film as a physical environment. Ann. Rev. Ecol. Syst. 7:161-176.

SURFACE MICROLAYERS OF THE NORTH ATLANTIC: MICROBIAL POPULATIONS, HETEROTROPHIC AND HYDROCARBONOCLASTIC ACTIVITIES

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ABSTRACT

The surface microlayer and subsurface water were sampled at 7 stations in the Georges Bank region of the North Atlantic during August 1977. Comparison of surface microlayer with subsurface data suggested that bacterial and ultraplankton direct counts, phytoplankton biomass, heterotrophic and hydrocarbonoclastic viable titers, and aliphatic hydrocarbon concentrations were enriched in the surface microlayer at several stations. This was demonstrated using the paired-t test (95% confidence level). Glutamate mineralization in the surface microlayers was significantly lower than in the subsurface water. Dodecane mineralization rates at 5°C and hydrocarbonoclastic viable counts covaried significantly (95% confidence level) with aliphatic hydrocarbon concentrations in surface microlayers. In surface microlayer and subsurface water, acetate and glutamate mineralization at 5°C covaried with acetate and glutamate mineralization at 20°C, respectively. Evidence obtained during one season's sampling of North Atlantic surface microlayer and subsurface waters indicated the enrichment of several microbial parameters in the surface microlayer. Hydrocarbon-degrading activity was not enriched. However, the hydrocarbonoclastic viable count and aliphatic hydrocarbon concentration were enriched in the surface microlayer.

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INTRODUCTION

Garrett (5) and Harvey (6) developed procedures for sampling the surface microlayers of the sea over a decade ago. Since that time only a few investigations have addressed the microbial communities of surface microlayers (2, 4, 15). In contrast, considerable attention has been drawn to the chemistry and physics of the sea-air interface (1, 10, 11, 12, 16, 17).

Despite the evidence that surface microlayers represent a chemically unique marine environment, there are few data to suggest that surface microlayers support unique microbial populations. In one study, lipolytic and proteolytic bacteria predominated over amylolytic isolates (15), while in another investigation, proteolytic and amylolytic activities were overwhelmingly preponderant over lipolytic and hydrocarbonolytic activities (4).

In the present study, surface microlayer and subsurface samples were collected from the North Atlantic. Microbiological and hydrocarbon data from surface microlayer and subsurface waters were compared and interrelationships among the parameters monitored are discussed.

MATERIALS AND METHODS

SAMPLING

Surface microlayer and subsurface samples were obtained at 7 stations in the Nantucket Shoals, Georges Bank region of the North Atlantic Outer Continental Shelf (NAOCS) during the last week of August 1977 (Fig. 1, Table 1). Sampling was conducted from a rubber boat positioned several hundred yards upwind of the mother ship to avoid contamination. Surface microlayers were collected with a 16-mesh stainless steel sampler (5). Subsurface microbiological samples were collected with a sterile, 2-liter bag sampler (General Oceanics). Subsurface samples for hydrocarbon analysis and microbial activities were collected with a 5-liter, Teflon-lined Go-Flo bottle (General Oceanics).

MICROBIOLOGICAL ANALYSIS

The ATP technique of Holm-Hansen and Booth (8) was used to determine microbial biomass on a 100 ml aliquant from each sample. Phytoplankton biomass was calculated from chlorophyll concentrations which were determined fluorometrically (9) on 100 ml of each sample. The lipopolysaccharide (LPS) assay of Watson et al. (19) was used to quantify bacterial biomass in a 10 ml aliquant of sample.

Bacterial and ultraplanktonic direct counts were determined by the fluorescence direct count (FDC) technique (19). A 10- and 100-ml aliquant from each sample was preserved in a final concentration of 0.1% (w/v) glutaraldehyde for bacterial and phytoplankton direct counts, respectively.

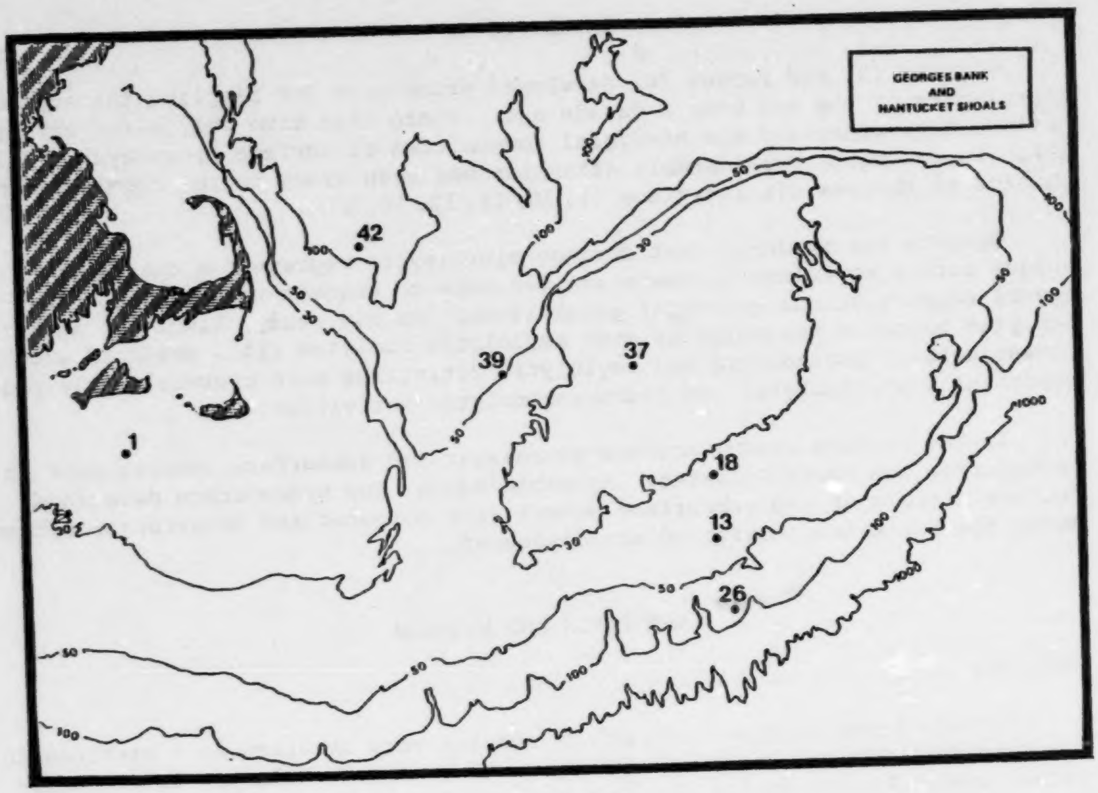

Figure 1. North Atlantic Outer Continental Shelf surface microlayer and subsurface sampling stations, late August 1977.

TABLE 1. SURFACE MICROLAYER STATIONS IN LATE AUGUST 1977

Station	Location	Depth (m)
1	41°06'N, 70°33'W	39
13	40°42'N, 67°36'W	85
18	40°59'N, 67°35'W	70
26	40°27'N, 67°30'W	135
37	41°27'N, 68°00'W	40
39	41°20'N, 68°44'W	95
42	41°51'N, 69°30'W	195

The membrane filter technique was used to obtain heterotrophic viable counts. Dilutions (or volumes) of seawater estimated to yield 30 to 100 colonies per filter were filtered through 47 mm 0.2 µm porosity Nuclepore membranes. The membranes were placed on marine agar (Difco) plates and incubated at 20°C for 2 weeks. After the original plates were counted, colonies were replicated onto hydrocarbon agar (13). Those appearing on the replica plates were scored as presumptive hydrocarbon utilizers.

Mineralization experiments were based upon the procedure of Wright and Hobbie (20) as modified by Hobbie and Crawford (7). Ten 50-ml aliquots from each samples were placed in sterile 100 ml serum bottles. The bottles were capped with septum caps fitted with Kontes reaction cups. Whatman No. 1 filter paper accordions (1 x 2 cm) saturated with 0.2 ml *b*-phenethylamine were in the Kontes cups. Four of the bottles were poisoned with 0.1 ml 0.005 M $HgCl_2$ solution. There was one trapping efficiency bottle control and a control for each nutrient. The trapping efficiency bottle was incubated with 0.5 ml ^{14}C-$HaHCO_3$ (specific activity [S.A.], 0.25 μCi/ml). Each nutrient was injected into the control and two test bottles. Glutamate and acetate were injected in 0.5 ml quantities (S.A. = 0.25 μCi/ml). Bottles received 0.1 ml n-(1-^{14}C) dodecane (S.A. = 0.25 μCi/ml). The control and one test bottle were incubated in the dark at 5°C, and the third bottle was incubated at 20°C. Populations in glutamate and acetate bottles were killed with 0.5 ml 50% trichloracetic acid after 4 h incubation. Populations in dodecane bottles were killed after 2 weeks. After $^{14}CO_2$ had been trapped, filter papers were transferred to scintillation vials. Aquasol scintillation cocktail (15 ml) was added to each vial, and $^{14}CO_2$ was quantified by liquid scintillation counting. Mineralization was computed from the fraction of the injected radioactivity that was trapped in the *b*-phenethylamine-saturated filter paper.

HYDROCARBON ANALYSIS

Hydrocarbon fractions were extracted and separated from seawater samples, then analyzed by gas chromatography.

STATISTICAL ANALYSIS

Paired-T tests and correlation coefficients (r) were computed using prepared statistical programs on a Monroe desk calculator. Critical values for T and r were obtained from Rohlf and Sokal (14).

RESULTS

The microbiology data obtained from the 7 stations are summarized in Tables 2-5. It is apparent from Table 2 that bacteria were approximately three orders of magnitude more abundant than ultraplankton in the surface microlayers. Bacterial and ultraplankton direct counts and viable counts were uniform over the geographic region. Biomass data demonstrated considerably more variation over Georges Bank and Nantucket Shoals. Microbial biomass in four of the seven surface microlayer samples was roughly equal to the sum of the bacterial and phytoplankton biomasses. This relationship was not consistent, and in the subsurface waters (Table 3), applied to Station 42 alone.

The hydrocarbonoclastic viable counts generally represented about 10% of the heterotrophic viable counts in both surface microlayer and subsurface

TABLE 2. MICROBIOLOGICAL DATA FROM NORTH ATLANTIC OUTER CONTINENTAL SHELF SURFACE MICROLAYERS IN LATE AUGUST 1977

Station	Direct count (cells/m^3)		Biomass (μg C/m^3)			Viable count (CFU/m^3, 20°C)	
	Bacterial (x 10^{11})	Ultraplankton (x 10^9)	Bacterial	Phytoplankton	Microbial	Heterotrophic (x 10^7)	Hydrocarbonolytic (x 10^6)
1	1.54	1.92	11.4	36.5	35.6	6.0	41.0
13	9.12	2.01	2.99	18.3	68.3	2.9	2.0
18	9.06	1.05	11.4	8.9	22.0	2.0	2.5
26	8.24	0.772	9.68	7.8	20.0	2.4	3.0
37	9.82	1.04	10.6	47.9	65.3	2.0	4.1
39	16.2	1.17	15.6	50.4	3.45	3.0	5.0
42	12.6	1.04	18.5	29.0	41.5	0.55	1.0

TABLE 3. MICROBIOLOGICAL DATA FROM NORTH ATLANTIC OUTER CONTINENTAL SHELF SUBSURFACE WATER IN LATE AUGUST 1977

Station	Direct count (cells/m^3)		Biomass (μg C/m^3)			Viable count (CFU/m^3, 20°C)	
	Bacterial (x 10^{11})	Ultraplankton (x 10^8)	Bacterial	Phytoplankton	Microbial	Heterotrophic (x 10^7)	Hydrocarbonolytic (x 10^6)
1	12.6	9.15	10.5	26.8	73.3	4.7	20
13	2.62	11.4	2.63	12.0	234	0.6	1.3
18	7.22	7.68	12.6	8.90	105	1.4	3.7
26	5.94	3.78	6.48	4.20	21.5	1.1	7.0
37	6.88	8.76	12.6	22.9	14.3	0.65	2.0
39	15.4	10.3	18.7	46.6	27.0	1.0	6.0
42	86.0	9.09	18.2	18.6	31.5	0.15	0.8

TABLE 4. MICROBIAL ACTIVITY AS INDICATED BY MINERALIZATION RATE IN NORTH ATLANTIC OUTER CONTINENTAL SHELF SURFACE MICROLAYERS, IN LATE AUGUST 1977 (µg C mineralized/m^3 per day)[a]

Station	Acetate substrate		Glutamate substrate		Dodecane substrate	
	5°C[b]	20°C	5°C	20°C	5°C	20°C
1	1.15×10^4	6.35×10^3	1.87×10^1	3.78×10^4	4.13×10^2	1.78×10^2
13	LTC	LTC	LTC	1.95×10^4	ND	ND
18	LTC	5.60×10^4	5.50×10^3	3.55×10^4	1.01×10^2	2.62×10^2
26	51.5	5.35×10^3	LTC	2.51×10^3	1.81×10^2	49.0
37	LTC	4.64×10^3	LTC	1.02×10^4	2.06×10^2	1.07×10^2
39	LTC	LTC	1.01×10^4	1.54×10^4	LTC	LTC
42	4.14×10^5	4.23×10^5	5.95×10^4	8.65×10^4	LTC	LTC

[a] ND = no data; LTC = less than control.

[b] Incubation temperature.

TABLE 5. MICROBIAL ACTIVITY AS INDICATED BY MINERALIZATION RATE IN NORTH ATLANTIC OUTER CONTINENTAL SHELF SUBSURFACE WATERS, IN LATE AUGUST 1977 (µg C mineralized/m^3 per day)[a]

Station	Acetate substrate		Glutamate substrate		Dodecane substrate	
	5°C[b]	20°C	5°C	20°C	5°C	20°C
1	LTC	LTC	2.47×10^4	9.20×10^4	3.26×10^2	3.50×10^2
13	LTC	4.76×10^3	1.57×10^4	3.06×10^4	ND	ND
18	2.03×10^4	1.73×10^4	1.05×10^4	1.66×10^4	2.19×10^2	1.21×10^2
26	2.45×10^3	3.58×10^3	LTC	3.73×10^3	7.75	LTC
37	5.95×10^3	3.05×10^4	9.95×10^3	2.83×10^4	7.96×10^2	1.23×10^3
39	2.34×10^4	1.49×10^4	2.60×10^4	5.80×10^4	LTC	LTC
42	4.48×10^5	4.45×10^5	8.25×10^4	1.30×10^5	LTC	LTC

[a] ND = no data; LTC = less than control.

[b] Incubation temperature.

samples. These estimates are probably an order of magnitude high since the figures used were from the hydrocarbon replica plates. Only 10% of the colonies originally isolated from the hydrocarbon replica plates grew in hydrocarbon broth or mineralized dodecane (data not shown).

Surface water temperatures during the late August 1977 cruise ranged from 14°C to 23°C. During early spring and winter months the temperature ranged from 4°C to 20°C and 0.2°C to 7°C, respectively. To determine whether Georges Bank microbial communities were adapted to colder temperatures, mineralization rates were determined at 5°C and 20°C. Overall, mineralization rates for acetate, glutamate, and dodecane in the surface microlayers (Table 4) and subsurface waters (Table 5) were low. At a number of stations, mineralization rates in poisoned controls exceeded rates in test bottles. There were no significant differences between 5°C and 20°C mineralization rates for any of the substrates tested.

The surface microlayer and near-surface data were analyzed to determine whether any geographic trends could be identified. Bacterial and ultraplankton direct counts and bacterial biomass were generally above average on the northwest edge of Georges Bank. Station 1, south of Nantucket Shoals, had above average bacterial direct counts, microbial biomass, heterotrophic and hydrocarbonolytic viable counts, and glutamate and dodecane mineralization rates in the subsurface sample. These parameters were not above average in the surface microlayer sample at that station. Most of the microbial parameters showed no concentration or activities trends over Georges Bank.

The primary reason for collecting surface microlayer and subsurface samples at each station was to determine whether the microbial population in the microlayer differed significantly from that in the underlying water. Surface microlayer and subsurface data from the 7 stations were compared by computing the ratios for each parameter. The numbers in Table 6 resulted from dividing surface microlayer data by subsurface data. Numbers in boldface type were for stations where confidence limits for surface microlayer and subsurface data did not overlap, and where the surface microlayer was enriched. Only three parameters, i.e., ultraplankton direct count, phytoplankton biomass, and heterotrophic viable count, were enriched significantly in the surface microlayer at half of the stations or more. Microbial biomass and glutamate mineralization at 20°C appeared to be enriched in the subsurface waters.

To substantiate the conclusions inferred from the data in Table 6, the paired-T statistic was computed for each parameter. The results appear in Table 7. As predicted, ultraplankton direct counts, phytoplankton biomass, and heterotrophic viable counts were significantly ($p = 0.05$) higher in Georges Bank surface microlayers than in subsurface waters. Glutamate mineralization at 20°C was depressed in the surface microlayers. Differences between the surface microlayers and subsurface waters were not significant ($p = 0.05$) for the other parameters measured.

Having demonstrated that the surface microlayers were not significantly enriched over the subsurface waters with respect to most of the parameters monitored, the next step was to determine whether the geographic distribution of each parameter differed between the two sample classes. This was tested by computing correlation coefficients for each parameter. As shown in Table 8, surface microlayer data covaried significantly with subsurface data for all parameters except bacterial direct counts and microbial biomass. This strongly suggests that factors affecting microbial populations in subsurface waters are the same as those at the surface microlayer.

TABLE 6. COMPARISON OF NORTH ATLANTIC SURFACE MICROLAYERS AND SUBSURFACE WATERS IN LATE AUGUST 1977: RELATIVE ENRICHMENT OF SURFACE MICROLAYER AT EACH STATION[a,b]

Parameter	Station						
	1	13	18	26	37	39	42
Bacterial direct count	0.12	$\underline{3.48}$	1.25	1.39	1.42	1.05	$\underline{1.46}$
Ultraplankton direct count	$\underline{2.10}$	$\underline{1.76}$	1.38	$\underline{2.04}$	1.19	1.19	0.11
Bacterial biomass	1.08	1.14	0.90	1.49	0.84	0.08	1.02
Phytoplankton biomass	$\underline{1.36}$	1.52	1.00	$\underline{1.86}$	$\underline{2.09}$	1.08	$\underline{1.56}$
Microbial biomass	0.48	0.29	0.21	0.93	$\underline{4.57}$	0.13	1.32
Heterotrophic viable count	1.50	$\underline{4.60}$	1.46	$\underline{2.16}$	$\underline{3.09}$	$\underline{2.99}$	$\underline{3.67}$
Hydrocarbonolytic viable count	$\underline{2.00}$	1.50	0.67	0.43	$\underline{2.05}$	0.83	1.25
Acetate mineralization:							
5°C	>	LTC	>	0.02	<	<	0.92
20°C	>	<	$\underline{3.24}$	$\underline{1.49}$	0.15	<	0.95
Glutamate mineralization:							
5°C	0.76	<	0.52	LTC	<	0.39	0.72
20°C	0.41	0.77	$\underline{2.14}$	0.07	0.36	0.26	0.66
Dodecane mineralization:							
5°C	$\underline{1.27}$	ND	0.46	$\underline{23.30}$	0.26	<	LTC
20°C	0.51	ND	$\underline{2.16}$	>	0.09	LTC	LTC

[a] Figures represent ratio of surface microlayer value/subsurface value; >, < is used where one of the values was less than control, i.e., > if subsurface value was less than control (LTC); LTC = both values were less than control; ND = no data.

[b] Underlined numerals indicate significant (0.95 confidence level) enrichment in surface microlayer.

The 13 parameters used in this study to describe the microbial populations of Georges Bank surface microlayers and subsurface waters could be divided into four groups of parameters. These were direct counts, biomasses, viable counts, and mineralization activities. Although each group described a different aspect of the microbial population, it was considered likely that there were significant relationships among the individual parameters. This hypothesis was tested by computing correlation matrices for the surface microlayer and subsurface data sets.

Complete data sets were obtained for 8 of the 13 parameters. Parameters for which any station data were missing were omitted from the correlation matrices. The matrices for the surface microlayers and subsurface waters are presented in Tables 9 and 10, respectively.

TABLE 7. COMPARISON OF NORTH ATLANTIC OUTER CONTINENTAL SHELF SURFACE MICROLAYERS AND SUBSURFACE WATERS IN LATE AUGUST 1977: SUMMARY OF PAIRED-T STATISTICS

Parameter	Mean values[a]		T_{obs}
	Surface microlayer	Subsurface	T_{crit} 0.95 = 1.94
Bacterial direct count (cells/m^3)	9.51×10^{11}	8.46×10^{11}	0.49
Ultraplankton direct count (cells/m^3)	1.29×10^{9}	8.59×10^{8}	3.11
Bacterial biomass (LPS-C/m^3)	10.0	11.7	-1.13
Phytoplankton biomass (chla-C/m^3)	28.4	20.0	2.72
Microbial biomass (ATP-C/m^3)	36.6	72.4	-1.34
Heterotrophic viable count (CFU/m^3)	2.7×10^{7}	1.4×10^{7}	5.23
Hydrocarbonoclastic viable count (CFU/m^3)	8.51×10^{6}	6.82×10^{6}	0.71
Glutamate mineralization at 20°C (µg C mineralized/m^3 per day)	2.96×10^{4}	5.61×10^{4}	-2.80

[a] Mean values presented are means for seven stations over Georges Bank.

TABLE 8. COMPARISON OF NORTH ATLANTIC OUTER CONTINENTAL SHELF SURFACE MICROLAYERS AND SUBSURFACE WATERS IN LATE AUGUST 1977: CORRELATION COEFFICIENTS (r) BETWEEN MICROLAYER AND SUBSURFACE DATA

Parameter	r	Significant correlation at $p = 0.05$
Bacterial direct count	0.16	No
Ultraplankton direct count	0.67	Yes
Bacterial biomass	0.93	Yes
Phytoplankton biomass	0.88	Yes
Microbial biomass	0.45	No
Heterotrophic viable count	0.91	Yes
Hydrocarbonoclastic viable count	0.95	Yes
Glutamate mineralization at 20°C	0.80	Yes

There were significant ($p = 0.05$) correlations between bacterial direct counts and both types of viable counts in the surface microlayers (Table 9). Ultraplankton direct counts also correlated well with viable counts. The strong correlation between phytoplankton biomass and hydrocarbonoclastic viable counts may have been due to increased concentrations of hydrocarbons such as pristine, produced by the phytoplankton. Much more data would be needed to demonstrate that this was not coincidental. Glutamate mineralization rates of 20°C were correlated to bacterial biomass, but not to either bacterial direct counts or viable counts. It was noteworthy that microbial biomass was not correlated to any other parameter.

TABLE 9. CORRELATION MATRIX FOR MICROBIAL PARAMETERS IN SURFACE MICROLAYERS OF NORTH ATLANTIC OUTER CONTINENTAL SHELF IN LATE AUGUST 1977[a]

Parameter	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Bacterial direct count	1.00							
Ultraplankton direct count	--	1.00						
Bacterial biomass	--	--	1.00					
Phytoplankton biomass	--	--	--	1.00				
Microbial biomass	--	--	--	--	1.00			
Heterotrophic viable count	0.68	0.67	--	--	--	1.00		
Hydrocarbonolytic viable count	0.76	0.56	--	0.88	--	0.89	1.00	
Glutamate mineralization at 20°C	--	--	0.60	--	--	--	--	1.00

[a] Only correlation coefficients significant at P = 0.05 are shown.

TABLE 10. CORRELATION MATRIX FOR MICROBIAL PARAMETERS IN SUBSURFACE WATERS OF NORTH ATLANTIC OUTER CONTINENTAL SHELF IN LATE AUGUST 1977[a]

Parameter	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Bacterial direct count	1.00							
Ultraplankton direct count	--	1.00						
Bacterial biomass	0.72	--	1.00					
Phytoplankton biomass	0.84	0.54	0.63	1.00				
Microbial biomass	0.53	--	0.66	--	1.00			
Heterotrophic viable count	--	--	--	--	--	1.00		
Hydrocarbonolytic viable count	0.53	--	--	--	--	0.97	1.00	
Glutamate mineralization at 20°C	0.63	--	--	--	--	0.85	0.92	1.00

[a] Only correlation coefficients significant at P = 0.05 are shown.

There were more significant (p = 0.05) relationships in subsurface waters than in the surface microlayers (Table 10). Bacterial, phytoplankton, and microbial biomasses; hydrocarbonoclastic viable counts and glutamate mineralization all correlated significantly with bacterial direct counts. The correlation between bacterial direct count and heterotrophic viable count was just below the 95% confidence level cutoff. However, the 0.97 coefficient between hydrocarbonoclastic and heterotrophic viable counts suggested that the latter might be included in the group of parameters covarying with bacterial direct counts. In contrast to Table 9, subsurface glutamate mineralization at 20°C covaried with bacterial direct counts and viable counts, but not with bacterial biomass. Bacteria apparently comprised a larger fraction of the subsurface microbial population as compared with the surface microlayer since microbial biomass covaried significantly with bacterial biomass and bacterial direct count in the subsurface waters.

DISCUSSION

Bacteria and ultraplankton concentrations in Georges Bank surface microlayers and subsurface waters were comparable to concentrations found in other regions (4, 18). Viable heterotrophs were significantly more abundant in surface microlayers than in subsurface waters; however, the concentration differences reported here were not as great as those reported for estuarine samples (3). This may have been due to the differences in sampling methods.

Crow et al. (3) collected surface microlayer samples by adsorption onto 47-mm diameter Nuclepore membrane filters. Each filter adsorbed 5.9 µl of sample. The sample thickness calculated from this would have been approximately 3.5 µm. The screen sampler collected a layer between 150 and 200 µm thick, or almost 50 times the thickness of the layer sampled using the membrane technique. If our surface microlayer data were multiplied by the dilution factor, as recommended by Sieburth et al. (16), then the differences between surface microlayer and subsurface populations over Georges Bank would be of the same magnitude as those reported for Gulf of Mexico estuaries (3).

Although there were more viable heterotrophs in the surface microlayers than in the subsurface water, mineralization rates for acetate, glutamate, and dodecane were not elevated in surface microlayer samples. The surface community was possibly incorporating the substrates at a higher rate than was the subsurface community. Unfortunately, only mineralization activity was monitored.

Similar to that of Taguchi and Nakajima (18), our data to some degree supports the concept that the surface microlayer is a unique habitat in the marine environment, and deserves more thorough investigation. In contract to Sieburth et al. (16) we did not find the surface microlayer to be enriched over the subsurface water with respect to every parameter monitored. The two regimes were distinguishable for five of the nine parameters, and four of those five parameters were enriched in the surface microlayers.

Recently P. Boehm (personal communication) observed that gas chromatographic profiles of aliphatic and aromatic hydrocarbon fractions in surface microlayers were distinguishable from those in subsurface waters. We demonstrated correlations between dodecane mineralization, hydrocarbonoclastic viable counts, and total aliphatic hydrocarbons in both classes of samples. However, more comparative bacteriological data are needed to determine whether the compositional profiles of the complex hydrocarbon mixtures in the surface microlayers and subsurface waters.

There were a number of interesting relationships between the two habitats and among parameters monitored. Taguchi and Nakajima (18) suggested that in estuarine systems the relationships showed seasonality. Sieburth (15) demonstrated that functionally, surface microlayer bacterial communities could be distinguished from subsurface communities. The surface communities had higher ratios of lipolytic and peptidolytic isolates than did the subsurface community, reflecting the distinctive chemical composition of the surface microlayer (1, 11).

More extensive, collaborative field investigations will be needed to verify the existence and describe the dynamics of microbial communities in surface microlayers.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Baier, R. E., D. W. Goupil, S. Perlmutter, and R. King. 1974. Dominant chemical composition of sea-surface films, natural slicks and foams. J. Rech. Atmos. 8:571-600.

2. Carlucci, A. F., and P. M. Williams. 1965. Concentration of bacteria from sea water by bubble scavenging. J. Cons. Perm. Int. Explor. Mer 30:28-33.

3. Crow, S. A., D. G. Ahearn, W. L. Cook, and A. W. Bourquin. 1975. Densities of bacteria and fungi in coastal surface films as determined by a membrane-adsorption procedure. Limnol. Oceanogr. 20:644-645.

4. Crow, S. A., D. G. Ahearn, W. L. Cook, and A. W. Bourquin. 1976. Microbial populations in coastal surface slicks, p. 93-98. *In* J. M. Sharpley and A. M. Kaplan (eds.), Proceedings of the Third International Biodegradation Symposium. Applied Sciences, London.

5. Garrett, W. D. 1965. Collection of slick-forming materials from the sea surface. Limnol. Oceanogr. 10:602-605.

6. Harvey, G. W. 1966. Microlayer collection from the sea surface: a new method and initial results. Limnol. Oceanogr. 11:608-613.

7. Hobbie, J. C., and C. C. Crawford. 1969. Respiration corrections for bacterial uptake of dissolved organic compounds in natural waters. Limnol. Oceanogr. 14:528-532.

8. Holm-Hansen, O., and C. R. Booth. 1966. The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnol. Oceanogr. 11:510-519.

9. Holm-Hansen, O., C. J. Lorenzen, R. W. Holmes, and J. D. H. Strickland. 1965. Fluorometric determination of chlorophyll. J. Cons. Perm. Int. Explor. Mer 30:3-15.

10. Hunter, K. A., and P. S. Liss. 1977. The input of organic material to the oceans: air-sea interactions and the organic chemical composition of the sea surface. Mar. Chem. 5:361-379.

11. Larsson, K., G. Odham, and A. Sodergren. 1974. On lipid surface films on the sea. I. A simple method for sampling and studies of composition. Mar. Chem. 2:49-57.

12. MacIntyre, F. 1974. The top millimeter of the coean. Sci. Amer. 230:62-77.

13. Makula, R., and W. R. Finnerty. 1969. Microbial assimilation of hydrocarbons. I. Fatty acids derived from normal alkanes. J. Bacteriol. 95:2102-2107.

14. Rohlf, F. J., and R. R. Sokal. 1969. Statistical tables. W. H. Freeman, San Francisco.

15. Sieburth, J. McN. 1971. Distribution and activity of oceanic bacteria. Deep-Sea Res. 18:1111-1121.

16. Sieburth, J. McN., P. J. Willis, K. M. Johnson, C. M. Burney, D. M. Lavoie, K. R. Hinga, D. A. Caron, F. W. French III, P. W. Johnson, and P. G. Davis. 1976. Dissolved organic matter and heterotrophic microneuston in the surface microlayers of the North Atlantic. Science 194:1415-1418.

17. Sutcliffe, W. H., Jr., E. R. Baylor, and D. W. Menzel. 1963. Sea surface chemistry and Langmuir circulation. Deep-Sea Res. 10:233-243.

18. Taguchi, S., and K. Nakajima. 1971. Plankton and seston in the sea surface of three inlets of Japan. Bull. Plank. Soc. Japan 18:20-36.

19. Watson, S. W., T. J. Novitsky, H. Quinby, and F. Valois. 1977. Techniques for measuring bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. 33:940-946.

20. Wright, R. T., and J. E. Hobbie. 1966. The use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology 47:447-464.

INTERFACES AND THE AQUATIC ENVIRONMENT

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ABSTRACT

Interfacial phenomena are extremely important to microorganisms metabolizing substrates which are immiscible with water. This paper reviews examples of biosurfactants produced by microorganisms to facilitate growth under these conditions. The most commonly observed biosurfactants are glycolipids containing rehalose, rhamnose, sophorose or other sugars. There are also examples of lipopeptides, phospholipids, carboxylic acids and neutral lipids. In many cases, microorganisms produce mixtures of surface active lipids.

There are many compartments in the aquatic environment, and the concept of compartmentalization includes the interfaces which exist in all habitats of the environment. Interfaces are of a scale comparable to that of microbes and possess a defined and active microbial population. These interfaces are between either gas-liquid, liquid-liquid, solid-liquid or gas-liquid-solid phases, and are a focal point for microbial degradation of a wide variety of insoluble pollutants.

Interfacial phenomena are complex and the topic is too broad to review here. Recent monographs by Marshall (26), Van Oss et al. (36) and Zviaginsev (40) provide considerable detail and background for those being introduced to the topic.

Microbes attach to bubbles, liquid droplets and solid surfaces, wherein problems are encountered relating to substrate solubilization (e.g., oil, sulfur, minerals, synthetics). Solution of these difficulties often involves biosynthesis of surface-active molecules which aid in solubilization, emulsification or wetting of the substrate, or in the attachment of the cell to an insoluble substrate.

Since considerable quantities of oil find their way into the aquatic environment, this paper focuses on the water-oil interface and the biosurfactants produced by microbes which utilize and degrade oil.

WATER-OIL INTERFACES

Liquid-liquid systems indicate that physically the system is biphasic and that one of the liquids, probably containing substrate or nutrients, is insoluble in the dominant phase. Most of the microbial studies with biphasic liquid systems center around water and oil. The mechanisms by which cells take up hydrocarbon is still not clear; however, some interfacial events that take place have been elucidated.

Aiba et al. (1), using *Candida guilliermondii* grown on n-alkanes, showed adsorption of oil droplet to cells. Addition of a surface-active agent gave improved emulsification but prevented contact, and low growth was observed. Mimura et al. (29) firmly established that cells utilizing hydrocarbons possessed an affinity for them while those not using hydrocarbons did not. Wang and Ochoa (37) demonstrated the importance of the interfacial area of the oil to the specific growth of *Candida intermedia* on n-hexadecane. Others have shown the importance of oil droplet surface area to growth. Improved surface area can be obtained by improved agitation, by addition of the proper surfactant, or by natural surfactants synthesized by the microbe. To demonstrate the role of the interface in such systems, Yoshida et al. (38) observed the association of *Candida tropicalis* cells with hexadecane under various fermentation conditions. Figure 1 shows *C rynebacterium lepus* cells imbedded in a matrix of bioemulsifier (see Gerson et al. [12]). *Candida tropicalis* cells surrounded by droplets of hexadecane were observed by Einsele et al. (9). The size limitation problem for metabolism of hydrocarbons still remains open to study. Microbes that adhere to oil droplets grow almost equally as well as those that accommodate small droplets on their cell surfaces. Two interfacial mechanisms appear to be important in hydrocarbon metabolism: growth on the hydrocarbon drop (drop-form) and accommodation of hydrocarbon droplets on the cell surface (accommodation-form) where the cell adsorbs micro-droplets of oil. A review of these mechanisms and the interfacial phenomena governing them is given by Gerson and Zajic (11).

Bacteria may not utilize exactly the same mechanisms as yeasts in adsorbing hydrocarbons. Marshall and Cruikshank (27) have grown *Flexibacter aurantiacus* and *Hyphomicrobium vulgare* in an oil-water system and both cultures tend to arrange themselves at right angles to the interface. Examination of the bulk water phase also shows floc formation. The flocs were of a defined structure which have been conveniently labelled "rosettes." The latter are astonishingly similar in appearance, but not scale, to the micelles described for surface-active agents.

The microbial affinity for the aqueous or oil phase is similar to another characteristic of synthetic surface-active agents. These agents are often characterized by their hydrophilic-lipophilic balance (HLB), i.e., their tendency to stay in the water phase or bind to the oil phase. Hydrocarbon-utilizing microbes may ultimately be analyzed in terms of their HLB characteristics, which can be determined by measurements of oil-water partition coefficients, contact angles and interfacial free energies.

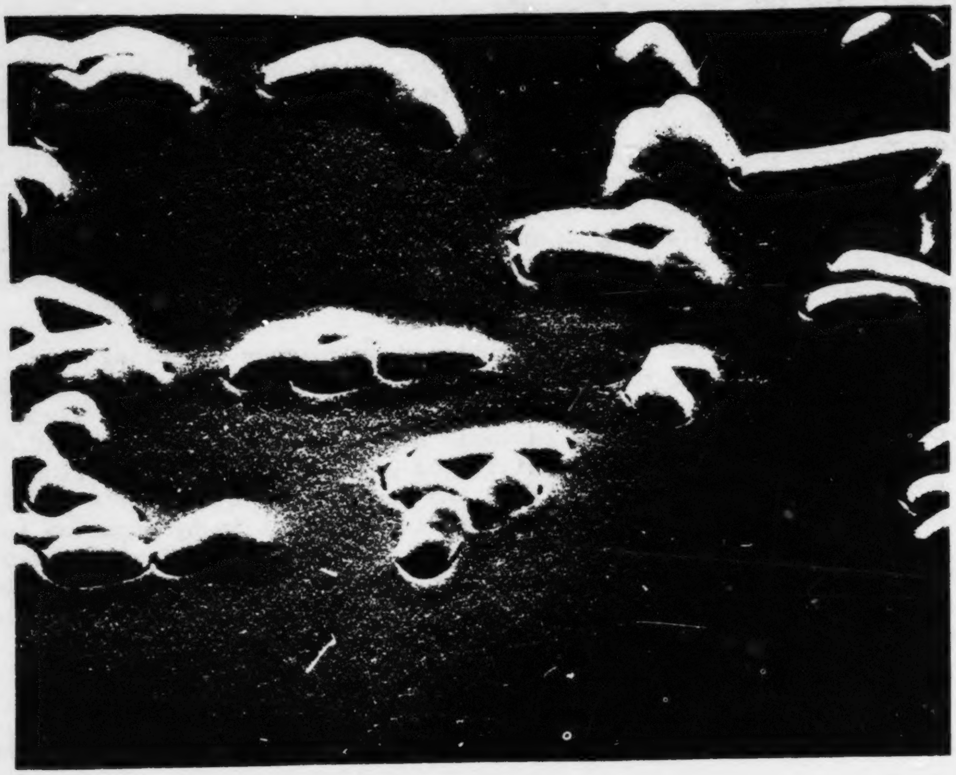


Figure 1. *Corynebacterium lepus* cells imbedded in a matrix of bioemulsifier.

SURFACE-ACTIVE AGENTS

In considering the aquatic environment, both synthetic and biosurfactants should be discussed. However, since biosurfactants are conceptually a more recent topic and give an explanation of how microbes resolve the handling of certain types of insoluble substrates and liquid-liquid interfaces, attention is given to microbial surfactants in this section. Microbial surfactants have been reviewed by Zajic and Panchal (39) and Gerson et al. (12).

There are a wide variety of extracellular surface-active agents produced by microorganisms. The most thoroughly characterized have been the glycolipids which fall into three major classes, all containing a hydroxy fatty acid covalently bonded to a carbohydrate. These are: lipids of trehalose and other carbohydrates with a fatty acid acyl bond; lipids of rhamnose bonded to the fatty acid by the hydroxyl group; and lipids of sophorose also bonded to the fatty acid by the hydroxyl group. Another class of surface-active agents are lipopeptides, which have a peptide component (usually less than 10 amino acids) and a fatty acid component. Other types of surface-active agents are phospholipids and neutral lipids, the latter including fatty acids, glycerides, alcohols and cerides.

TREHALOSE LIPIDS AND RELATED COMPOUNDS

When *Arthrobacter paraffineus* KY4303 was grown on *n*-paraffin as the sole source of carbon, it produced a glyco-lipid with strong emulsifying properties which was isolated and incompletely characterized. Each molecule of glycolipid contained a disaccharide (trehalose) and two β-hydroxy-α-branched fatty acids esterified at undetermined sites on the sugar. The fatty acids had a general formula R_1-CH(OH)-CHR_2-COOH, where $R_1 = C_{18}$ to C_{23} and $R_2 = C_7$ to C_{12}. Glycolipids containing trehalose have been isolated from several other species of *Arthrobacter* as well as from *Mycobacterium, Brevibacterium, Corynebacterium* and *Nocardia* when grown on *n*-paraffins (33,31,16,2).

It was suggested that the aforementioned ubiquitous glycolipid was produced by bacteria and used to emulsify the hydrocarbon phase into the aqueous phase. This conclusion is supported by several observations. The glycolipids isolated from at least two of the bacteria have been shown to have good emulsifying properties (34,31). The addition of penicillin to *A. paraffinus* blocked the formation of the glycolipid and depressed the rate of *n*-paraffin consumption indicating that the glycolipid is important to hydrocarbon assimilation (33). When a species of *Nocardia* was grown on glycerol, there was only a trace of a trehalose lipid (31). However, it was produced in good yield when *n*-paraffins were the substrate. Furthermore, additions of glycolipid to the paraffin media caused a marked enhancement in initial cell growth (Table 1).

TABLE 1. EFFECT OF TREHALOSE LIPID ON GROWTH ON *Nocardia* SP. ON *n*-PARAFFIN[a]

% Lipid added	Dry wt (g/l) at 23 h
0.0	1.90
0.0001	2.10
0.001	2.70
0.01	3.87

[a] From Rapp and Wagner (31).

An interesting effect on the glycolipids is observed when the appropriate bacteria were grown on sucrose or fructose instead of *n*-paraffins (32,16). The carbohydrate portion, trehalose, of the glycolipid was replaced by sucrose or fructose. Thus, when *A. paraffineus* KY4303 was grown on sucrose, two glycolipids were isolated. One was identified as 6-O-glucosyl-β-fructoside corynomycolate (β-hydroxy-α-branched fatty acid), while the other was an ester of sucrose and two molecules of corynomycolic acid. When fructose was the sole carbon source, two different glycolipids were isolated, fructose-6-corynomycolate and fructose-1,6-dicorynomycolate. Similar results were obtained when species of *Corynebacterium, Nocardia* and *Mycobacterium* were grown on sucrose or fructose instead of *n*-paraffins.

RHAMNOLIPIDS

Edwards and Hayashi (8) completed the identification of a glycolipid which had been first isolated by Jarvis and Johnson (17) from the culture broth of *Pseudomonas aeruginosa* grown on glycerol. This glycolipid was composed of two molecules of rhamnose and two molecules of β-hydroxy-decanoic acid and has been given the trivial name of R-1 rhamnolipid. The full structure is 2-0-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Fig. 2A). It was later reported that when *P. aeruginosa* was grown on hydrocarbon as the sole carbon source, two rhamnolipids were isolated. One of these was identical to R-1 (Fig. 2A). The other was labelled R-2 and differed from R-1 in that it contained only one molecule of rhamnose (i.e., α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Fig. 2B).

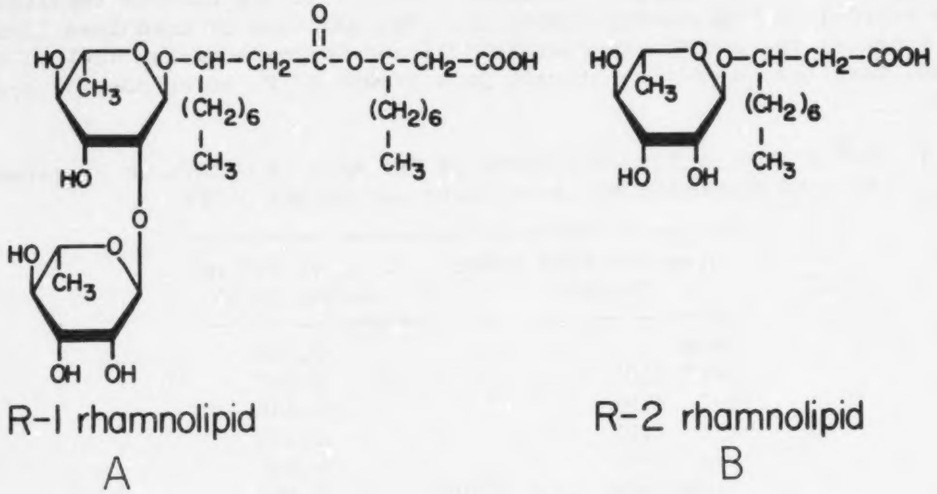


Figure 2. Two rhamnolipids isolated from growth of *Pseudomonas aeruginosa* on hydrocarbon as the sole carbon source.

Histasuka et al. (14) proposed that the rhamnolipids were produced by *Pseudomonas aeruginosa* as emulsifiers to disperse potential hydrocarbon substrated into the aqueous phase. Rhamnolipid R-1 was added in varying concentrations to *P. aeruginosa* S7B1 growing on 1% hexadecane. As concentration of the lipid was increased up to 0.005%, a dramatic increase occurred in the rate of growth of the bacterium. As the concentration of lipid was increased above 0.005% there was no further enhancement of the growth rate. This stimulation of synthetic surfactants (e.g., Noigen EA141 or Tween 20). Furthermore, neither the rhamnolipid nor the synthetic surfactants enhanced the rate of growth of *P. aeruginosa* on glucose. Thus, it was postulated that the rhamnolipid acted as an emulsifier, making the hydrocarbon substrate more readily available for utilization by the bacterium.

The surface activity and emulsifying power of the rhamnolipid were consistent with the above proposal. It caused a marked decrease in the surface tension of water (minimum 34 dynes/cm). Furthermore, the critical micelle concentration of about 0.005% corresponded to the concentration which resulted in the maximum growth stimulation of *P. aeruginosa* on hexadecane. The emulsion stability of hydrocarbon-water mixtures containing rhamnolipid was comparable to the synthetic surfactants.

The effect of rhamnolipid on the growth rate of other hydrocarbon-utilizing bacteria was also tested. All of the strains of *P. aeruginosa* tested showed a marked enhancement of growth. However, addition of rhamnolipid did not affect the growth rate of several species of *Corynebacterium, Achromobacter, Micrococcus* or *Bacillus*. Species specificity was also observed by Itoth and Suzuki (16), who isolated a mutant of *P. aeruginosa* (strain PU-1) which could not metabolize hydrocarbons and did not produce either the R-1 or R-2 rhamnolipids. The addition of either R-1 or R-2 to the culture resulted in growth of PU-1 on hydrocarbon (Table 2). The addition of trehalose lipids, which enhances the growth of *Nocardia* (31) and *Corynebacterium* (34) on hydrocarbons, resulted in only relatively poor growth of *P. aeruginosa* on hydrocarbon.

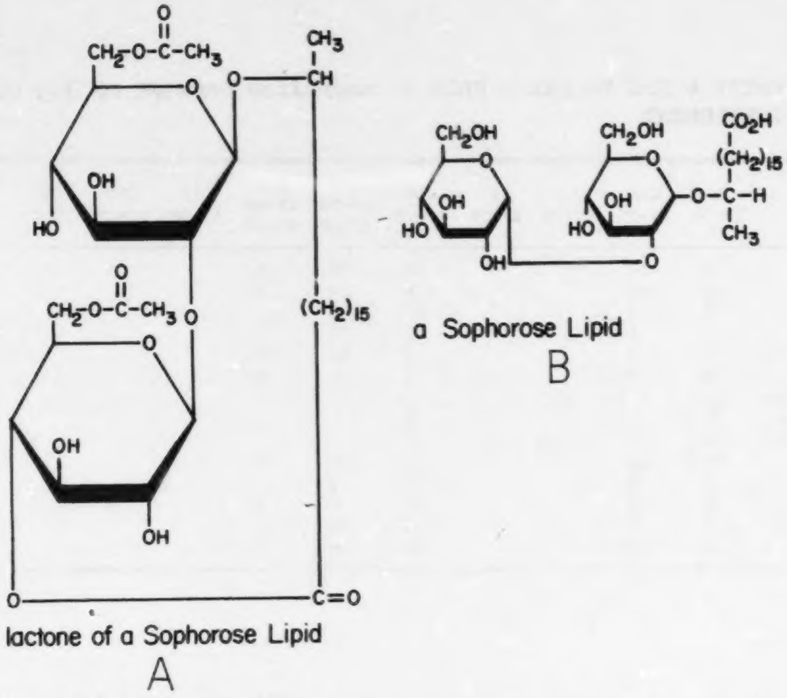
TABLE 2. THE EFFECT OF BIOSURFACTANTS ON THE RATE OF GROWTH OF *P. aeruginosa* PU-1 ON HYDROCARBONS (from Itoth and Suzuki [16])

Biosurfactant added (μg/ml)	O.D. at 660 mμ (after 50 h)
none	0.030
R-1[a] (10)	0.140
R-1 (50)	0.645
R-2[b] (10)	0.105
R-2 (50)	0.655
Trehalose lipid (100)	0.187
Trehalose lipid (100)	0.110

[a] Isolated from *Nocardium* sp. [b] Isolated from *Corynebacterium* sp.

SOPHOROSE LIPIDS

When *Torulopsis magnoliae* is grown on glucose (2.0% w/v) media, it produces substantial quantities of a water-insoluble oil (5% v/v) (13). Two major glycolipids were isolated from this oil, both of which contain the disaccharide sophorose and a hydroxy fatty acid (Fig. 3A,B). However, unlike the β-hydroxy fatty acids usually observed in glycolipids, the hydroxyl group is remote from the carboxylic function. The two types of fatty acids found, 17-L-hydroxyoctadecanoic acid and 17-L-hydroxy-octadec-9-enoic acid, were both covalently bonded to sophorose through the hydroxyl function.



lactone of a Sophorose Lipid
A

a Sophorose Lipid
B

Figure 3. Two major glycolipids isolated from a water-insoluble oil produced from growth of *Torulopsis magnoliae* on glucose media.

The yield of sophorose lipids from *T. magnoliae* was increased several fold by reducing the glucose concentration to 10% and supplementing the media with various esters of hydrocarbons (35). Furthermore, the nature of the supplement influenced the distribution of hydroxy fatty acids in the sophorose lipids (Table 3). When esters were added to the media, usually the major lipid found contained the hydrolyzed fatty acid with a hydroxyl function on the penultimate or terminal carbon. However, there was a limit to this effect, and most of the fatty acids found were limited to 16-19 carbon atoms.

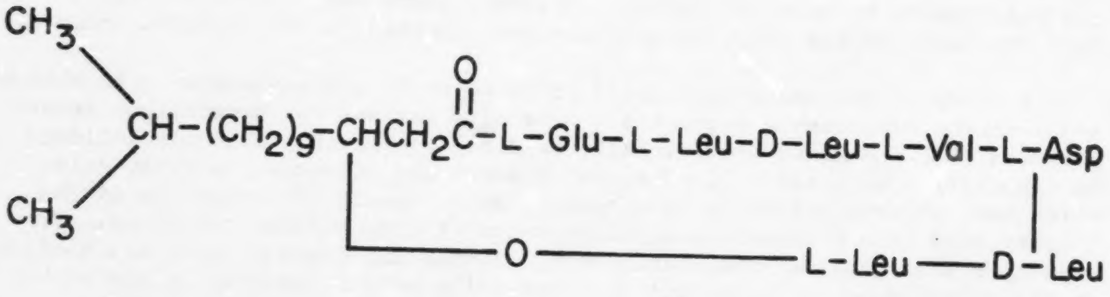
A study of the sophorose lipids produced by *T. gropengiesseri* with alkane and 1-alkene supplements suggested a metabolic pathway for hydrocarbon incorporation (20). Alkanes are initially oxidized to terminal alcohols followed by dehydration to 1-alkenes. Terminal alkenes are converted to fatty acids which then undergo oxidation to a hydroxy acid. Rapid incorporation of the hydroxy acid into a sophorose lipid protects it from further oxidation. *T. magnoliae* also produces small amounts of crystalline diacetyl lactones similar to the oily sophorose lipids but with the fatty acid esterified to the sophorose (Fig. 3B) (35). A study of *T. gropengiesseri*, which produces larger amounts of these cyclic glycolipids, showed that their production was induced by hydrocarbon supplements (18).

TABLE 3. FATTY ACIDS ISOLATED FROM *T. magnoliae* GROWING ON 10% GLUCOSE PLUS SUPPLEMENT

Supplement	C16 15-OH	C16 16-OH	C17 16-OH	C17 17-OH	C18 17-OH	C18 17-OH 9C = C	C18 18-OH 9C = C	C19 18-OH	C20 19-OH	C21 20-OH	Others	Relative total yield
None	9	10			21	50	6		4			16
Palmitate	40	40			5	14	1					73
Stearate	2	2			80	9					8	68
Oleate	2	2			6	77	13					85
11-Eicosenoate	2	3			9	56	11				19	52
Erucate	2	4			5	65	12				12	51
Heptadecanoate			87	13								64
Nonadecanoate	3	5	24	9	6			53				63
Heneicosanoate	4	9	54	17	5			8		3		60
Hexadecane	36	43			5	13	2				1	56
Ectadecane	2	4			85	4					5	55
Eicosane	8	7			57	8	2		14		4	54
Docosane	8	9			67	9	3				4	64
Tetracosane	8	8			63	16	5					51

LIPOPEPTIDES

Lipopeptides have been isolated from a wide variety of bacteria and yeasts, but only a few have been thoroughly characterized. The best known example is a crystalline compound produced by *Bacillus subtilis* and which has been given two trivial names, surfactin or subtilysin. This contains a peptide chain of seven amino acids in a cyclic compound with a β-hydroxy fatty acid (Fig. 4) (21). The compound shows strong surface activity (3,6), and a 0.005% aqueous solution has a surface tension of only 28 dynes/cm, compared with surface tension of pure water of 72 dynes/cm at 20°C. This lipopeptide has several properties which have been attributed to this surface activity. It causes lysis of the membranes of mammalian erythrocytes, protoplasts and spheroplasts of several bacteria, and also inhibits blood clotting.



Surfactin or Subtilysin

Figure 4. Crystalline compound produced by *Bacillus subtilis*.

An emulsifying factor has been isolated from *Candida petrophilum* on hydrocarbon (15). This compound has not been completely characterized but contains glutamic acid, aspartic acid, alanine and leucine, and an unidentified fatty acid. When *C. petrophilum* was grown on glucose the lipopeptide was not found. If this emulsifying factor were added to *C. petrophilum* growing on hydrocarbons, the rate of growth was enhanced in direct proportion to the amount added. The properties of an extracellular surfactant produced by *C. lipolytica* were studied by Gerson et al. (10).

Several other lipopeptides have been isolated from microbial broths. These include lipids containing β-hydroxy fatty acids isolated from *Serratia marsescens, Pseudomonas viscoas, Bacillus mesentericus* and *Nocardia asteroides* (4,7). Less common are lipopeptides containing a simple fatty acid, such as that isolated from *Mycobacterium paratuberculosis* (23).

PHOSPHOLIPIDS

There are relatively few examples of appreciable amounts of phospholipids being isolated from cell-free microbial broths. During growth of *Thiobacillus thiooxidans* and concomitant elemental sulphur oxidation, Jones and Benson (19) noticed a drop in the surface tension of the broth which they attributed to the phospholipids which could be isolated from the broth. Several different phospholipids were found (Table 4), but the major components were phosphatidyl glycerol and phosphatidic acid. It was later shown that each of the isolated phospholipids had the ability to wet elemental sulphur (5).

TABLE 4. PHOSPHOLIPIDS ISOLATED FROM *Thiobacillus thiooxidans*[a]

	% of Total cellular	% of Total extracellular
Phosphatidyl glycerol	75	49
Phosphatidyl choline	25	17
Phosphatidyl ethanolamine	3	--
Phosphatidyl inositol	--	1
Diphosphatidyl glycerol	--	5
Phosphatidic acid	--	29

[a] From Jones and Benson (19).

When *Corynebacterium alkanolyticum* was grown on hexadecane, it produced a small amount of extracellular phospholipids (1.9 mg/dl). However, the addition of 50 μg/ml penicillin to the culture greatly increased the amount of extracellular phospholipid to 49.4 mg/dl. The phospholipids produced by *C. alkanolyticum* were also found to be sensitive to the hydrocarbon substrate (22). When grown on hexadecane, the constituent fatty acids of the phospholipids were mainly C14, C16, C18 and C20. However, when pentadecane was the substrate, the fatty acids were mainly C13, C15, C17 and C19. Extracellular emulsifiers were also isolated from *Corynebacterium* sp., strain PPS-II, by Panchall and Zajic (30).

NEUTRAL LIPIDS

Originally the surface-active agents produced by *Thiobacillus thiooxidans* were thought to be only phospholipids (19). A later study showed the presence of neutral lipids in the extracellular broth (5). These included hydrocarbons (22%), triglycerides (4%), fatty acids (4%), hydroxy fatty acids (30%), monoglycerides (3%) and others (31%). Like the phospholipids, these all had the ability to wet sulphur and may aid in bacterial attachment to sulphur surfaces.

Extracellular neutral lipids are often induced by using hydrocarbons as a substrate. A species of *Acinetobacter* produced very little extracellular lipid when grown on nutrient broth/yeast extract but yielded a substantial amount of neutral lipid on hexadecane (Table 5 [25]). Similarly, the production of fatty acids by *Micrococcus cerificans* is enhanced by using hydrocarbon media (Table 6 [24]).

TABLE 5. EXTRACELLULAR LIPIDS PRODUCED BY *Acinetobacter* SP. (μmol/l) (from Makula et al. [25])

Lipid class	Nutrient broth/ yeast extract	Hexadecane
Phospholipids	0	0
Triglycerides	2.4	25.6
Mono- and diglycerides	0	410.0
Fatty acids	4	60
Fatty alcohols	0	0.5
Wax esters	0	2800.0

TABLE 6. FATTY ACIDS PRODUCED BY *Micrococcus cerificans* (from Makula and Finnerty [24])

Medium used	Total Fatty Acid Produced	
	Cellular (μmol/g)	Extracellular (μmol/l)
Acetate	9.3	5.0
Hexadecane	8.2	60.5
Heptadecane	8.4	49.0
1-Hexadecane	8.6	25.8

SUMMARY

Larger microbial cells such as yeast accommodate small oil droplets which attach to the cell surface. In bacteria this process is often reversed and the bacteria may adsorb to the surface of oil droplets and grow in a fashion referred to as "drop form." Attachment of cells to oil, or oil to cells, depends upon the hydrophilic-lipophilic characteristics of the cell surface and whether either biosurfactants are synthesized or surfactants are added to give oil emulsification.

Some cells align themselves at interfaces. Rosette formation of cells is observed in the aqueous medium which resembles micelle formation. Cultures of *Flexibacter* and *Hyphomicrobium* readily show rosette formation.

A wide variety of surfactants are synthesized by microbes. The glycolipids fall into three classes, i.e., lipids of trehalose, lipids of rhamnose, and lipids of sophorose. Another class of biosurfactants are the lipopeptides in which the peptide component usually contains 10 amino acids or less. Other types of surface-active agents are phospholipids and neutral lipids, the latter including fatty acids, glycerides, alcohols and cerides. Microbial surfactants offer a new mechanism and a new horizon in our understanding of microbial interfaces.

LITERATURE CITED

1. Aiba, S., V. Moritz, J. Someya and K. L. Haung. 1969. Cultivation of yeast cells by using n-alkanes as the sole carbon source. J. Ferm. Technol. 47:203-210.

2. Anderson, R. J., and M. S. Newman. 1933. The chemistry of the lipids of the *Tubercle bacillus*. J. Biol. Chem. 101:499-504.

3. Arima, K., A. Kakinuma, and G. Tamura. 1968. Surfactin, a crystalline peptide-lipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhbition of fibrin clot formation. Biochem. Biophys. Res. Commun. 31:488-494.

4. Asselineau, J. 1966. The bacterial lipids. Hermann, Paris. 372 pp.

5. Beebe, J. L., and W. W. Umbreit. 1971. Extracellular lipid of *Thiobacillus thiooxidans*. J. Bacteriol. 108:612-614.

6. Bernheimer, A. W., and L. S. Avigad. 1970. Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. J. Gen. Microbiol. 61:361-369.

7. Cason, J., and T. S. Prout. 1948. Concerning the structure of phthioic acid. J. Am. Chem. Soc. 70:879-880.

8. Edwards, J. R., and J. A. Hagashi. 1965. Structure of a rhamnolipid from *Pseudomonas aeruginosa*. Arch. Biochem. Biophys. 3:415-421.

9. Einsele, A., H. Schneider, and A. Fiechter. 1975. Characterization of micro-emulsions in a hydrocarbon fermentation by electronmicroscopy. J. Ferm. Technol. 53:241-243.

10. Gerson, D. F., J. E. Zajic, and A. Margaritis. 1976. Microbial separation of bitumen from Athabasca tar sand. Univ. Western Ont. Press. 241 pp.

11. Gerson, D. F., and J. E. Zajic. 1978. Surfactant production from hydrocarbons by *Corynebacterium lepus* sp. nov. and *Pseudomonas asphaltenicus* sp. nov. Dev. Ind. Microbiol. 19:577-599.

12. Gerson, D. F., J. E. Zajic, D. G. Cooper, and M. D. Ouchi. 1978. Microbial separation of bitumen from Athabasca tar sand, Part II. Univ. Western Ont. Press. 316 pp.

13. Gorin, P. A. J., F. T. Spencer, and A. P. Tullock. 1961. Hydroxy fatty acid glycosides of sophorose from *Torulopsis magnoliae*. Can. J. Chem. 39:846-855.

14. Hisatsuka, K., T. Nakahara, N. Sano, and K. Yamada. 1971. Formation of rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. Agr. Biol. Chem. 35:686-692.

15. Iguchi, T., I. Takeda, and Ohsawa. 1969. Emulsifying factor of hydrocarbon produced by a hydrocarbon-assimilating yeast. Agr. Biol. Chem. 33:1657-1658.

16. Itoth, S., and T. Suzuki. 1972. Effect of rhamnolipid on growth of *Pseudomonas aeruginosa* mutant deficient in n-paraffin-utilizing ability. Agric. Biol. Chem. 36:2233-2235.

17. Jarvis, T. G., and J. J. Johnson. 1949. A glyco-lipid produced by *Pseudomonas aeruginosa*. J. Am. Chem. Soc. 71:4124-4126.

18. Jones, D. F. 1967. Novel macrocyclic glycolipids from *Torulopsis gropengaseri*. J. Chem. Soc., p. 479-484.

19. Jones, G. G., and A. A. Benson. 1965. Phosphatidyl glycerol in *Thiobacillus thiooxidans*. J. Bacteriol. 89:260-261.

20. Jones, D. F., and R. Howe. 1968. Microbiological oxidation of long-chain aliphatic compounds. Part I: Alkanes and alk-1-enes. J. Chem. Soc., p. 2801-2808.

21. Kakinuma, A., M. Hori, M. Isono, D. Tanura, and K. Arima. 1969. Determination of amino acid sequence in surfactin, a crystalline peptide-lipid surfactant produced by *Bacillus subtilis*. Agr. Biol. Chem. 33:971-972.

22. Kikuchi, M., T. Kanamaru, and G. Nakao. 1973. Relation between the extracellular accumulation of L-glutamic acid and the excretion of phospholipids by penicillin-treated *Corynebacterium alkanolyticum*. Agr. Biol. Chem. 37:2405-2408.

23. Laneelle, G., and J. Asselineau. 1962. Isolement de peptido-lipides à partir de *Mycobacterium paratuberculosis*. Biochim. Biophys. Acta 59:731-732.

24. Makula, R. A., and W. R. Finnerty. 1972. Microbial assimilation of hydrocarbon: cellular distribution of fatty acids. J. Bacteriol. 112:398-407.

25. Makula, R. A., P. J. Lockwood, and W. R. Finnerty. 1975. Comparative analysis of the lipids of *Acinetobacter* species grown on hexadecane. J. Bacteriol. 121:250-258.

26. Marshall, K. C. 1976. Interfaces in microbial ecology. Harvard Univ. Press. 165 pp.

27. Marshall, K. C., and R. H. Cruickshank. 1973. Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. Arch. Mikrobiol. 91:29-40.

28. Marshall, K. C., R. Stout, and R. Mitchell. 1971. Mechanisms of initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. 68:337-348.

29. Mimura, A., S. Watanabe, and I. Takeda. 1971. Biochemical engineering analysis of hydrocarbon fermentation. J. Ferm. Technol. 49:255-271.

30. Panchal, C., and J. E. Zajic. 1978. Isolation of emulsifying agents from a species of *Corynebacterium*. Dev. Ind. Microbiol. 19:569-576.

31. Rapp, P., and F. Wagner. 1976. Formation of a trehalose lipid by *Nocardia* sp. grown on n-alkane. 6th Internat. Ferm. Sym. (Berlin), p. 63.

32. Suzuki, T., K. Tanaka, and S. Itoth. 1974. Sucrose lipids of *Arthrobacteria, Corynebacteria* and *Nocardia* grown on sucrose. Agr. Biol. Chem. 38:557-563.

33. Suzuki, T., K. Tanaka, and S. Kinoshita. 1969. The extracellular accumulation of trehalose and glucose by bacteria grown on n-alkanes. Agr. Biol. Chem. 33:190-195.

34. Suzuki, T., K. Tanaka, I. Matsubara, and S. Kinoshita. 1969. Trehalose lipid and α-branched-β-hydroxy fatty acid formed by a bacteria grown on n-alkanes. Agr. Biol. Chem. 33:1619-1627.

35. Tulloch, A. P., J. F. T. Spencer, and P. A. J. Gorin. 1962. The fermentation of long-chain compounds by *Torulopsis magnoliae*. Can. J. Chem. 40:1326-1338.

36. van Oss, C. J., C. F. Gillman, and A. W. Neumann. 1975. Phagocytic engulfment and cell adhesiveness. Dekker, New York. 160 pp.

37. Wang, D. I. C., and A. Ochoa. 1972. Measurements on the interfacial areas of hydrocarbon in yeast fermentations and relationship to specific growth rates. Biotech. Bioeng. 14:345-360.

38. Yoshida, F., T. Yamane, and H. Yagi. 1971. Mechanism of uptake of liquid hydrocarbons by microorganisms. Biotech. Bioeng. 8:215-228.

39. Zajic, J. E., and C. Panchal. 1976. Bioemulsifiers. CRC Crit. Rev. Microbiol. 5:39-66.

40. Zviaginsev, D. Z. 1973. The interaction of microorganisms with solid surfaces. University Press, Moscow. 175 pp.

DISCUSSION

COMPARTMENTALIZATION IN AQUATIC ENVIRONMENTS

PASSMAN: Dr. Poole, Jorgensen has been doing some work using radiolabelled sulfate, sulfide, etc., in polychromide tubes which she is sticking into sediment. Have you used this method, and can this data from a fairly inexpensive radiolabelled study be extrapolated to biodegradation, in general?

POOLE: Oh, yes, I'm a great believer in using labelled sulfate. She developed a rather cheap method for looking at sulfate reduction. At the University, we had no money, no fume hoods, we just couldn't do the distillation to separate the labelled sulfate and sulfide, but you can make it acid, then standardize the pressures and remove a gas sample. If you inject labelled sulfide into toluene at -7°C, you can therefore count it in a toluene cocktail cheaply. We can do 35 samples in half a day with the method. Using the standard method, we do three.

PRITCHARD: Are aromatic rings likely to be degraded in the anaerobic environment or more likely to be funneled back out into the aerobic water layer?

POOLE: I don't know. I think maybe a biochemist could better answer your question. But aromatics can be degraded in anaerobic environments.

RAYMOND: You said that sediments in cold areas may not be very active. Do you have some evidence on the effect of temperature on anaerobic biodegradation?

POOLE: Yes, we have some evidence that says during our winters with temperatures of 0-4°C in the sediments, there is very little biodegradation occurring.

RAYMOND: Do you suppose this is due to H_2S because normally H_2S is not more toxic at colder temperatures?

POOLE: No, actually it is more toxic at warmer temperatures.

DAGLEY: I would like to comment on the degradation of aromatic compounds in anaerobic environments. The subject has been reviewed extensively by W. C. Evans in Nature (London), Vol. 270, pp. 17-22.

TIEDJE: Dr. Lichtenstein, I'm concerned with what you are following, maybe a lot of these products are standard metabolic intermediates which are no longer of concern in the toxicology sense.

LICHTENSTEIN: This could be possible. With a ^{14}C label you can follow the fate of the compound. I stated that we should be concerned about the parent compound and the biologically active metabolites. You first have to determine what is present, then which of the compounds is biologically active. If you obtain soluble ^{14}C compounds in water, it is probably no longer insecticidal to mosquito larvae. Toxic to mosquito larvae does not mean that the compounds are also toxic to humans. The biological activity of the benzene-soluble compounds toward insects is relatively easy to determine. I agree, we have to determine what the materials are and if they are biologically active. We are restricted, of course, because we can neither conduct tests with human beings nor with each of the 1,400,000 species of insects.

MATSUMURA: I wasn't too sure what you meant when you said the microorganisms were responsible for this increased binding. Do you mean that aminoparathion was formed by microorganisms and thereby increasing binding?

LICHTENSTEIN: Aminoparathion is not found in sterilized soil, nor do we encounter binding phenomena. Based on data with sterilized soil, reductive processes in soil are dependent on microorganisms. This, however, is not true for all compounds but appears to be so with compounds containing nitro-groups. We repeated these tests with Dyfonate, another organophosphorous compound, utilizing irradiation and re-inoculation. The binding of Dyfonate is not related to the activity of microorganisms.

MATSUMURA: Have you ever changed the pH and attempted to re-extract the aminoparathion?

LICHTENSTEIN: Not in these experiments, where we purposely kept the soil within its natural state. We have tried to extract bound residues at different pH but we were unsuccessful. In this particular situation, we tried to keep the soil as natural as possible. We made six different extractions with various combinations of solvents. Usually we were able to extract 90% with the first three extractions.

LIU: How do you know that the bound material in the soil is one compound or two compounds or what? We have done some experiments with ^{14}C pesticides in soils. We fractionated the soil or organic matter, following the standard procedure, into six different fractions--α humus, β humus, etc. After a few weeks incubation, we extracted the major portion of the radioactivity from one of these six fractions. The bound material was a single compound which apparently chemically or physically attached to that soil organic matter. However, after 6 months incubation, the radioactivity was evenly distributed in the six different fractions. This is evidence, that gradually these radioactive compounds are being incorporated into the soil organic matter. It is no longer one metabolite. I'm suggesting the soil organic matter takes up the radioactivity and then goes to the oats. If you analyze the oats you will find this radioactivity is in amino acids, sugars, and other biological compounds.

LICHTENSTEIN: I am not sure if you asked me a question or if you wanted to add something. I think, though, that it would be a big contribution if one could repeat this experiment and concentrate on the oats. In a routine analytical laboratory one usually does not extract soil more than three times. All we have shown is that you don't extract everything and that a sizable portion of the ^{14}C was unextractable. Even in the case of parathion, we don't have direct proof of what is bound. We have indirect proof that only amino compounds are being bound and this process involves microorganisms. With other organophosphorous materials, residues are also bound but microorganisms do not appear to be involved.

BOLLAG: The problem of bound residues was discussed intensively in a symposium in Colorado. Unquestionably, it is one of the major problems in pesticide research today and, in general, when dealing with xenobiotic compounds. They may be bound in soil, but little is known as to how this occurs. I believe that microbial activities are very important in the binding of parathion. It is well known that amino compounds are quickly bound in soil. Some time ago we did an experiment where you added amino compounds to soil for 1/2 hour; when we tried to extract them, we found they were bound. I do not think, in this case, it is a biological binding but perhaps a chemical or physical binding.

LICHTENSTEIN: Once you have the amino compound.

BOLLAG: Yes. This is true if you have an amino compound and also if you have a phenol which may be formed. In my opinion, it is the activity of the microorganism which forms the intermediates, analinic compounds and phenolic compounds, and these may then be bound by physical or chemical factors within soils. It may be the clays or organic matter, etc., but that is usually difficult to determine. It is also possible to imagine binding through enzymes in soil which are active in formations of analines and phenolics.

GARNAS: Did you ever run a sterile control and put one of the reduction products into it?

LICHTENSTEIN: Yes, we added parathion to sterile soil, but no binding was observed under these conditions.

PRITCHARD: In relation to Dick Garnas' question, did you try sterilizing soil by gamma radiation in which you had bound pesticides and did you grow the oats on that sterilized soil?

LICHTENSTEIN: No, we didn't do that.

PRITCHARD: What you seem to infer is that the oats are responsible for bringing the aminoparathion out of the bound state, where, in fact, it might be the microbial populations which are responsible.

LICHTENSTEIN: This could be. We conducted studies with crop plants containing residues which had grown in soil treated with insecticide. Bound residues were found in some of the crop plants. In recent studies conducted at the Biotron under different climatic conditions, we found that binding is to some extent dependent on environmental conditions.

CHESTERS: I've got a general question that I would like to address to the panel. We have been talking about several unqiue microenvironments. It is pretty clear these unique zones in the aquatic environment appear to effect biodegradation. We talked about adsorption and sediment surfaces, we talked about surface microlayers, and we talked about anoxic environments. In light of the purpose of this conference, which is to suggest methodologies or strategies with a development of a protocol for examining toxic organic chemicals in the environment, would you, in reality, suggest that these unique zones be taken into account when devising the strategy? Should any of these unique microenvironments be included in that testing protocol? And, if so, how would you do it?

POOLE: Are we saying that the word unique suggests something special? We are talking about the marine environment, one part of the environment is surface layer, another part the anoxic, etc. I fail to see how you cannot take these areas into account. If you don't take them into account you are going to fail.

CHESTERS: How are you going to account for them?

POOLE: That's where I duck. Taking anoxic situations, I think you have to rely on microcosms. There are people here much more qualified in microcosms than I am. That is the easiest way to start studying the problem, then perhaps you want to go back into the environment for further study. You've got to relate the two.

CLESCERI: Dr. Poole, regarding your comment on the microcosm, I think something should be added. The microcosms originate from the so-called unique environment. Right? I mean, this is not something like your pet microcosm that's been in your laboratory for the past few years. I think this is an extremely important feature.

POOLE: Yes. The whole idea is to make it as realistic as possible. You are completely right. You've got to be as realistic as possible. You must allow for seasonal effects.

LICHTENSTEIN: I have a feeling that the word microcosm could get five different definitions from five people. We talked about microcosms for a whole week last year in Corvallis. Basically, people are trying to simulate what goes on in nature. Whatever we do, however, one has to realize that only restrictive answers to certain problems will be obtained. I cannot see that we will ever come up with a microcosm which would answer the whole multitude of questions we are facing. We will be rather limited, even if we should be able to come up with a good microcosm.

CHESTERS: It seems to me that we are talking about a lot of compounds. If we are going to make assessments of hazardous compounds, and they are going to be realistic, in the sense of those that are truly hazardous being declared hazardous, then our testing methodologies must not be so sensitive that everything becomes hazardous. We can't be talking about things that are going to cost more than the defense budget. We can't be talking about doing things that are going to require more personnel than are available in the history of the world. And how are we going to boil some of these unique facets that we've talked about here into some consensus opinion of how to proceed. Lichtenstein says we need more in the EPA budget.

CLESCERI: Dr. Passman, with respect to your last comment, if one of the pollutants has a very low density and accumulates at the surface, clearly then the biology of that surface is going to reflect that. Right?

PASSMAN: For example?

CLESCERI: I was thinking of oil.

PASSMAN: Well, oil has a gaseous fraction that is going to volatilize fairly quickly. A considerable portion of the non-volatile fractions are photo-oxidized in a fairly short order, and much of the remaining materials sink fairly quickly. Some of the work our colleagues have been doing on the Argo-Merchant spill and on several of the other New England spills show that oil disappears pretty quickly from the surface and either winds up in the sediments or is diluted throughout the water column. Two months after the Argo-Merchant spill, we could not identify Argo-Merchant oil in the sediment within 200 meters of the wreck.

CLESCERI: These time frames are long for that period. So clearly within those weeks or months that you are speaking of there is going to be a difference in the biological composition of that layer.

PASSMAN: Oh, true, from an esoteric perspective, I'd say the surface microlayers offer some fascinating areas of investigation. What we are addressing here is the question of whether EPA should be concerned with the microbial degradation of pollutants in the surface zone or other unique microenvironments. Again, we are talking about cost effectiveness of a certain type of study. When you address this question, as Dr. Chesters also mentioned, you've got to decide what needs to be studied and set priorities. From both our own data and that in the literature, I don't think there is sufficient rationalization for putting a high priority on studying the role of degradation in the surface microlayer, per se. I think the residence time in sediment is going to be much greater, and you should get more fruitful returns from investigating that area.

CLESCERI: Some of this is dependent upon what the ultimate test procedures are going to be.

PASSMAN: That's true. Again, we are talking about dollars and public health/public safety.

COLWELL: There really isn't any mystery in the association of hydrocarbonoclastic bacteria with surface film and finding a correlation with occurrence of phytoplankton. Plankton can contain fatty acids and various kinds of lipid-like materials, including aliphatics, etc. Such correlations have been recorded, particularly in marshes at the end of the fall season, where release of material occurs with hydrocarbons of biogenic origin increasingly entering the systems. I find it interesting that you make the statement that there is no need to worry about surface films because this is, in fact, an example of a microcosm, where mixtures of pollutants can have serious effects. For example, heavy metal and pesticide will concentrate in the surface film, with 3 or 4-hundred-thousand-fold concentration in the film, creating a significant effect on the bacteria in the film, as well as with their capability of dealing with whatever the pollutant may be.

PASSMAN: While I agree with you, the question becomes, what is the residence time of these materials in the film? How long will they continue to persist in the film before the film is broken, and they are either diluted into the water column or trapped in other micro habitats?

COLWELL: Well, that was one of the reasons we used a very large "microlayer," in fact a "surface layer," as was pointed out by the earlier speaker. We had to determine whether the bacteria in that mixed layer were the same in our study as in the air above the water. It is important to determine whether, in the turbulent surface layer, you are dealing with the same microorganisms.

FLOODGATE: There are two comments I would like to add, if I may. First of all, on the stability of that surface layer, it is much more stable than people imagine. It isn't something present only during flat calm seas. When you have a lot of breaking waves, the surface layer is reformed almost instantaneously. It is a very stable layer, and doesn't mix up nearly as much as you might imagine. Mixing occurs only in a few microns, perhaps tens of microns, on the surface. The next comment I would like to make is on this word "unique," which has been flying around with careless abandon. I think one must recall that Winogradsky, who was a better microbiologist than any of us here, said that he could isolate any bug he wanted from the sole of his boots. So you can find any bug you want from anywhere in the sea. So, it is true that you can find certain microorganisms in the surface layers and the same ones immediately below it. But that doesn't mean to say you shouldn't look in the surface layer to find out what is going on there.

DiGERONIMO: I have no working experience in the marine environment, but in the surface layer that you said should be discounted, would these microorganisms found there originate from the surface layer or do they originate from sediment? Do you think the populations found in the surface contribute to the subsurface layer?

PASSMAN: Your question is, are the microorganisms in surface films from aerosols, aerosols coming from special sources, or are they from resuspended sediments?

DiGERONIMO: You explained there was no difference between the subsurface layer and the surface layer. I was wondering if the lack of difference was because either the surface layer is being formed by the subsurface layer or the surface layer is contributing to the subsurface layer. I was wondering which is important. Can you comment on that?

PASSMAN: I can comment. We haven't looked at our taxonomic data, so I won't address whether the speciation differences are due to organisms coming from one source or another. Some of the work done by Carlucci in bubbling type experiments indicates that organisms originally suspended in the water will be differentially layered onto the surface due to size and affinity for organic constituents of the surface microlayer. So that says the original source was the water column, and they were selected out onto the surface layer because of bubbling or because of transport mechanisms. In the microlayer itself, a 0.1 micron layer, few or none of the organisms there are of the dimension where they can be totally contained by that monolayer. We are sampling the screen layer, 150-300 microns, and we know what their distribution is in that layer. This question could be addressed by using different types of sampling methods. For instance, I believe Al Bourquin's Nuclepore membrane method picked up a layer of 3.7 microns?

BOURQUIN: That's correct, depending upon the slick or film, of course.

NORKRANS: We have gotten one-micron thick samples using the Nuclepore membrane.

PASSMAN: What types of counts do you get on them? Are they of the same magnitude that we see on the screen? We get approximately 10^6-10^7 bacteria/ml. Using the Nuclepore membrane, we got approximately the same magnitude of cells. So chances are, then, it's safe to say the cells are existing right near that monolayer, probably protruding through the monolayer or existing directly below the monolayer, but you get approximately the same number of cells in the one-micron sample as you do in the 120-125 micron sample. Where do they come from? There are several sources.

DiGERONIMO: Have you investigated the areas for total activity? Is there a difference at this microlayer vs the sublayer? Is there more activity at the microlayer, or below it?

PASSMAN: We found the glutamate activity was depressed in the microlayer as compared with the subsurface water from approximately one meter. This is from one season of study, and I wouldn't want to make any pervasive statements suggesting this is how it is over the Georges Banks. I would expect to find some seasonality; I need a broader data base before saying specifically what's happening. During the late summer, however, we found glutamate mineralization depressed. We didn't look at incorporation. This may have been enriched in the surface microfilm. There were a number of limitations with the study.

NORKRANS: Did you make specific determinations of biomass?

PASSMAN: Not in that study. We did isolate several yeasts and fungi, and greater than 70% of the hydrocarbonolytic bacteria isolated grew on a complex mixture of approximately 20 aliphatic and aromatic hydrocarbons. The mineralization was strictly with 1-^{14}C n-dodecane. So we are studying just one n-alkane as opposed to a variety of alkane and aromatic hydrocarbons in mineralization. We were looking only at mineralization, not incorporation. I think there has been some work that indicates that cometabolism may be an important parameter. Studying the secondary metabolites might be the route for looking at hydrocarbon metabolism in natural systems.

BOLLAG: Are fungi included in total biomass?

PASSMAN: Yes.

BIODEGRADATION IN MICROCOSMS

Chairperson, F. Matsumura

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SYSTEM DESIGN FACTORS AFFECTING ENVIRONMENTAL FATE STUDIES IN MICROCOSMS[†]

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ABSTRACT

Two microcosms used in environmental fate studies are described. One is a static system which utilizes a sediment/water core and the other is a continuous-flow system using a structured sediment/water growth vessel with continuous addition of seawater. The effects of design characteristics of both systems on the fate of methyl parathion (MPS) was studied. Sediment/water cores taken directly from the environment were generally slower to degrade MPS than cores "structured" with sediment and water in the laboratory. Degradation rates were slower when sediment to water ratios were increased (water decreased) in either type core. Laboratory-aged cores were less reactive than "fresh" cores when $^{14}CO_2$ and degradation products of C^{14}-MPS was measured.

Continuous-flow microcosms which were acclimated to methyl parathion over a 50-day period were more active in removal from the water column and metabolism of MPS than aged systems not exposed to MPS for the preceding 25 days.

Total radioactivity measurements in the water of continuous-flow systems showed a greater removal from active systems than sterile ones. This is attributed primarily to sorption to sediments. Product analysis showed removal of MPS with production of aminomethyl parathion and $^{14}CO_2$, whereas the only product in the control system was p-nitro phenol.

This paper supports the contention that design features will partly determine the outcome of a fate experiment. Intact sediments, aged systems, acclimated

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[†] Contribution no.   from the Gulf Breeze Environmental Research Laboratory.

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systems and size of the microcoms were shown here to affect the data from two different microcosms.

INTRODUCTION

The setting of water quality criteria for pesticides and toxic substances requires the establishment of guidelines for the registration of these chemicals. Guidelines for aquatic environments require development of protocol in two major areas: toxicology, and fate and transport. Interaction between these two areas has become increasingly complex due to decreased use of very persistent polyhalogenated organic chemicals and an increased use of a variety of new chemicals which have a finite lifetime in the environment. Laboratory toxicological studies must now be continually scrutinized and evaluated according to the information generated on fate and transport. Since microbial degradation is one of the most important aspects of fate and transport, it is critical that good, representative methods be available to determine the biodegradation potential of the environment in question.

We recognize three key issues that influence an aquatic degradation study: [illegible] Due to the specialized nature of aquatic environments, terrestrial fate studies are not directly applicable. B) Methodology for studying the fate of chemicals in aquatic environments is seriously lacking. C) Field studies are more difficult to perform in aquatic environments than in terrestrial systems.

Consequently, our research has centered on the development of microcosm or model ecosystem technology to generate information on degradation processes in estuarine salt marsh environments. For simplicity, a microcosm is defined as a containerized portion of an aquatic environment maintained in the laboratory under controlled conditions. It is designed to simulate, but not necessarily duplicate, natural environmental situations. In the case of an estuarine system, characteristics such as sediment-water interfaces, high dilution capacity, and salinity changes should be modeled into the microcosm study.

It is also important to design systems for easy adaptation or assimilation into other research programs, particularly those that provide a data base for development of registration guidelines. The systems should be simple, reproducible, and inexpensive. Before establishing a system *a priori*, system design characteristics should be varied and tested to determine their effect relative to an established degradation process and to reveal possible deficiencies of the microcosm (9,4,22). The purpose of this paper is to describe two of our microcosm systems, one previously described by Bourquin et al. (5), and to indicate features that should be studied in detail before predictive capability can be realized.

MATERIALS AND METHODS

MICROCOSMS

Two different microcosms have been developed: one, referred to as "ecocore," uses cores of water and sediment; the second, referred to as a continuous-flow microcosm, uses a structured water/sediment system into which seawater and pesticide are fed continuously.

Eco-core microcosms (Fig. 1) were set up by obtaining an undisturbed (intact) core of sediment (50 g), water (200 ml), and a sterile glass tube (3.5 x 30 cm). Cores were taken from a local pristine salt marsh (10-17 ppt salinity, 24-28°C); the sediment cores were approximately 95% sand (by weight) with a 3-5 mm layer of fluffy, high organic content (15-20%) detritus on top. Cores were maintained at constant temperature (24°C±2). Aeration gave maximum mixing in the water column without resuspending the sediments.

For each eco-core experiment, the system was "spiked" with both radiolabelled and cold pesticides to give a final water concentration of 0.5 mg/liter. The distribution of parent compound and degradation products in the water and sediment was then followed with time. Water overlying the sediments was sampled (5 ml) and analyzed on a periodic basis; after a designated incubation period, the microcosm was dismantled and the sediments analyzed for parent compound, degradation products, and bound residues. Sterile controls (2% formalin) were run simultaneously. Air exiting each system was passed through polystyrene bead resin (Amberlite XAD-4) to monitor for volatile products, and radiolabelled CO_2 was trapped in aqueous sodium hydroxide (2N).

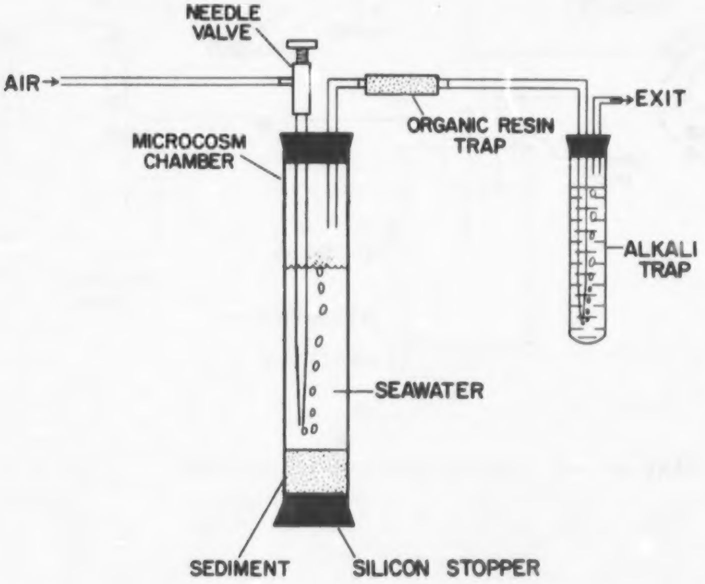

Figure 1. Schematic diagram of eco-core microcosm.

The continuous-flow microcosm (Fig. 2) consisted of a reactor vessel into which water (350 ml) and sediment (100 g) collected from the same salt marsh were placed. After the sediment had settled (∿2 h), a 3-4 cm layer of detritus settled over an equally thick layer of sand. Both fresh filtered seawater and sterile artificial seawater containing the radiolabelled pesticide were metered into the reactor vessel at equal rates. Turnover time for the contents of the reactor vessel was 25 h. Concentration of pesticide entering the vessel was 20 μg/liter; salinity was between 15 and 17 ppt. The system was continuously aerated for mixing and oxygenation (dissolved oxygen at sediment-water interface was 20% of saturation); an undisturbed sediment-water interface was maintained. Air exiting the system was monitored as described above. At periodic intervals, water samples (20 ml) and sediment cores (3 ml) were analyzed for parent compound and degradation products. Sterile controls were obtained by adding formalin (2% v/v, final concentration) to the original water-sediment mixture followed by the continuous addition of formalized (0.1%) artificial seawater. This was sufficient to eliminate viable bacteria in the water in the growth vessel even with the continuous addition of non-sterile seawater.

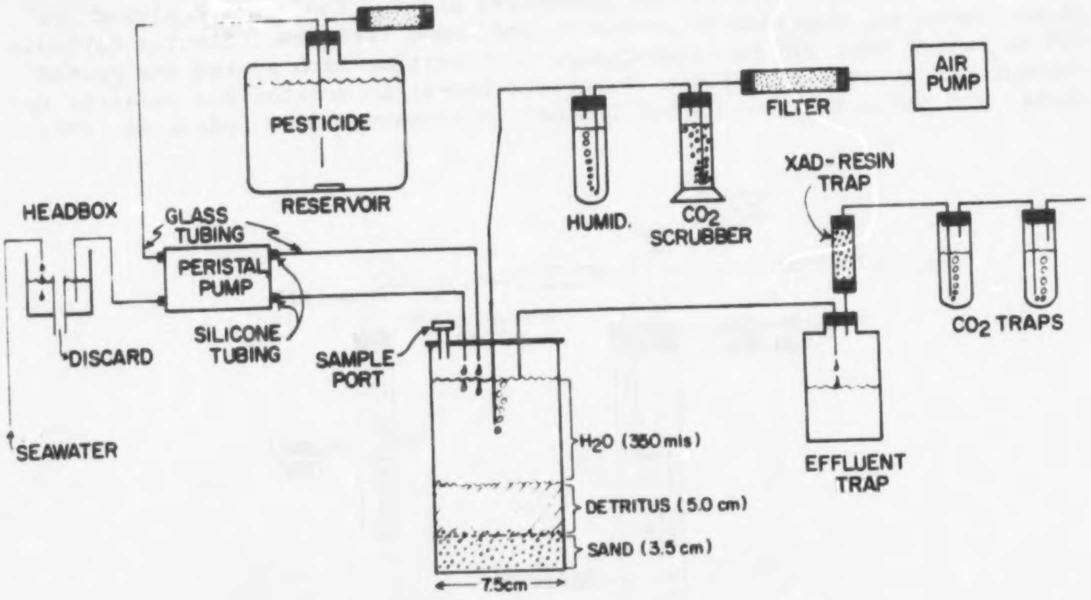

Figure 2. Schematic diagram of continuous-flow microcosm.

CHEMICALS

C-14 methyl parathion (2, 6 ring-labelled, specific activity = 30 Ci/mole, Amersham-serale Corp.) was the pesticide for these studies. It was the sole source of carbon for each microcosm except for the carrier (acetone), which was added at a final concentration of 1 mg/liter for eco-core microcosms and 40 μg/liter for the continuous flow microcosm. All other chemicals were nanograde purity.

ANALYTICAL

Figure 3 gives a schematic of the analytical methodology employed. All water samples were extracted with methylene chloride, twice at ambient pH (7.6) and twice under acidified (pH 2.0 with concentrated HCl) conditions. All sediment samples were washed twice by centrifuging and resuspending with water and then extracted four times with acetonitrile. Acetonitrile was back-extracted with methylene chloride and the latter sample checked for products. Radioactivity in all samples was measured with PCS^{TM} scintillation cocktail (Amersham Corp., Arlington Heights, Ill.).

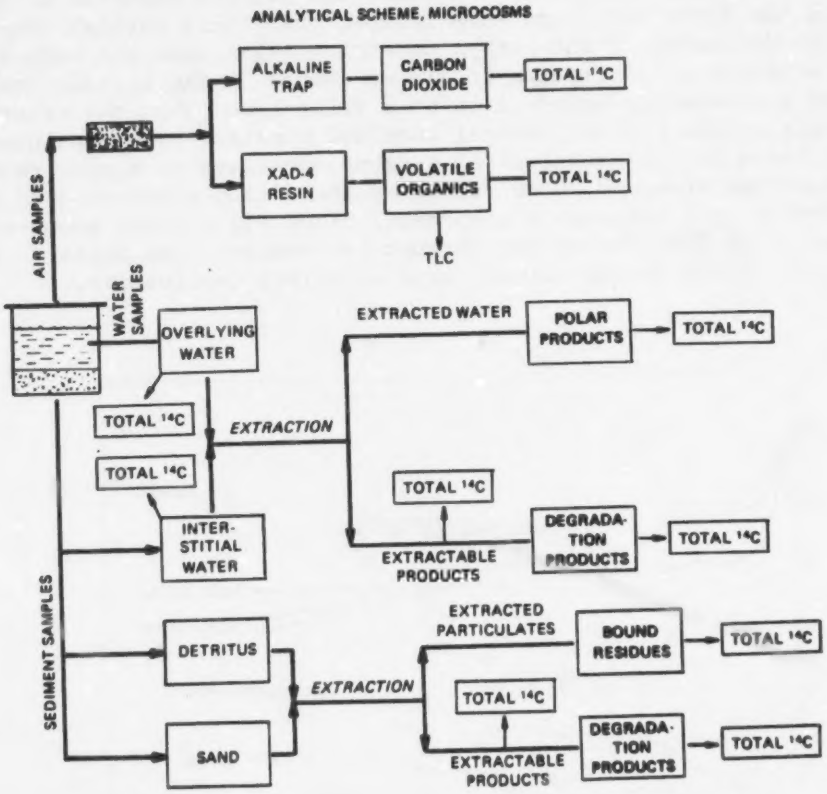

Figure 3. Analytical scheme for analysis of ^{14}C-pesticide fate in microcosms.

Methylene chloride extracts were chromatographed on silica gel thin layer plates by an ether:hexane (3:1, v:v) solvent system, and spots visualized by autoradiography in a spark chamber (Birchover Inst., Ltd., Letahworth, Herts, England). Degradation products were verified by co-chromatographing standards on a gas chromatograph equipped with a flame photometric detector by UV spectrometry and by mass spectrometry.

Radiolabelled carbon dioxide in alkaline traps was determined by scintillation counting of a 0.5 ml sample in Harvey's scintillation cocktail (Harvey Instrument Co.). All combustions were done with a Harvey Instrument Combustion Chamber at 900°C.

RESULTS

ECO-CORE

Chemical analyses of water overlying the sediment in an eco-core microcosm experiment are shown in Figure 4. Total radioactivity in the water (unextracted water) of both the experimental and the sterile controls dropped rapidly during the first 2-3 d and then leveled off. This initial drop was probably due to diffusion of the methyl parathion (MPS) into the sediments as there was no evidence of adsorption to glass. Water in the sterile control always reached a constant concentration at a value lower than the experimental. Total ^{14}C levels in the sterile control remained constant with continued incubation, while those in the experimental systems decreased at a slow rate. This steady decrease appeared to be caused by increasing adsorption to sediments (mediated by some biological component) since significant amounts of carbon were not lost from the system through degradation (see below). Duplicate experimental cores (dotted lines) gave excellent replication.

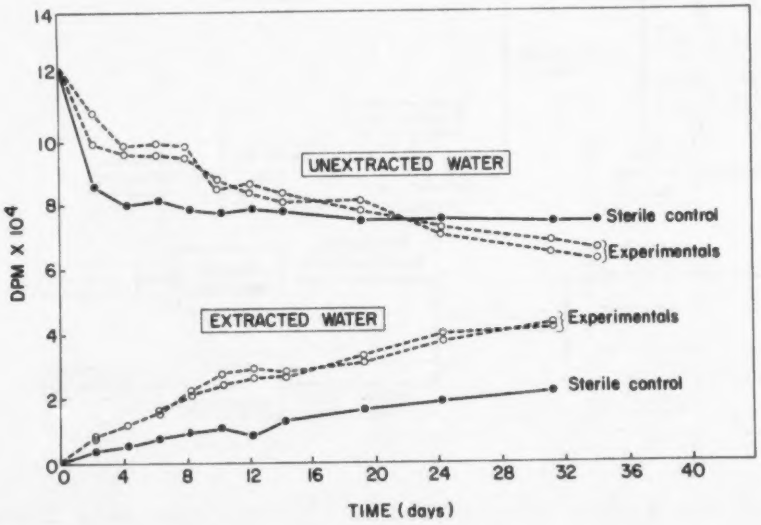

Figure 4. Radioactivity in the water column above sediment of an eco-core microcosm spiked with methyl parathion. Unextracted water represents total radioactivity in water sample (dotted lines are results from duplicate cores). Extracted water represents radioactivity remaining in water sample after solvent extraction (see Materials and Methods). Sterile control contained 2% formalin.

The radioactivity remaining in the water after solvent extraction (extracted water or polar products) increased steadily, above sterile control values, from day zero. This was the result of slow metabolism of the hydrolysis products of methyl parathion (MPS) to more polar unextractable materials. The presence of unextractable radioactivity in water from sterile controls was due to the incomplete extraction of paranitrophenol (see below) by methylene chloride. Unextractable products in experimental systems increased at a rate approximately equal to the loss of total radioactivity in the water column. All eco-core microcosms without sediment (water only) to date were incapable of generating polar products above sterile control levels or of inducing loss of total radioactivity from the water column. This indicates that components of the sediments may mediate the production of the polar unextractable products through the initial attack on MPS.

The fate of parent compound and the production of breakdown products in sterile and nonsterile microcosms are shown in Figures 5 and 6. MPS disappeared rapidly in the nonsterile systems; by day 12 only 0.1% of the original MPS remained. During the first 10 d of incubation, 80-90% of the loss was accounted for by the products produced in the water column, i.e., paraaminophenol (PAP), aminomethyl parathion (AMPS), polar unextractable products, and CO_2. Thereafter, the products accounted for only 50-70% of the loss of methyl parathion. The remaining 30-50% was bound to sediment (see below).

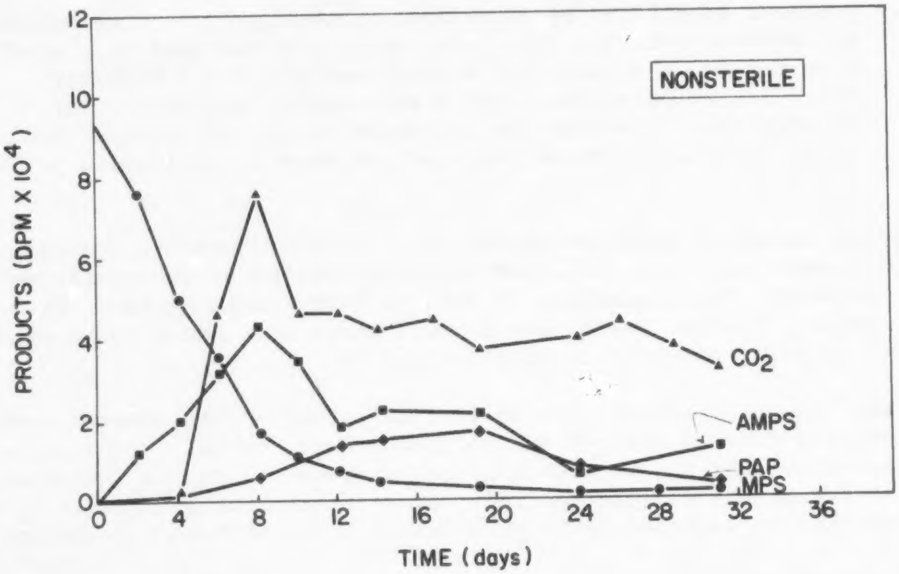

Figure 5. Products identified by thin-layer chromatography in methylene chloride extractable material from water above the sediment in a nonsterile eco-core microcosm spiked with methyl parathion (0.5 mg/liter). MPS = methyl parathion, AMPS = aminomethyl parathion, PAP = *p*-aminophenol. Products are expressed as dpm/ml of water column. $^{14}CO_2$ is total produced per collection period averaged per number of days in that period.

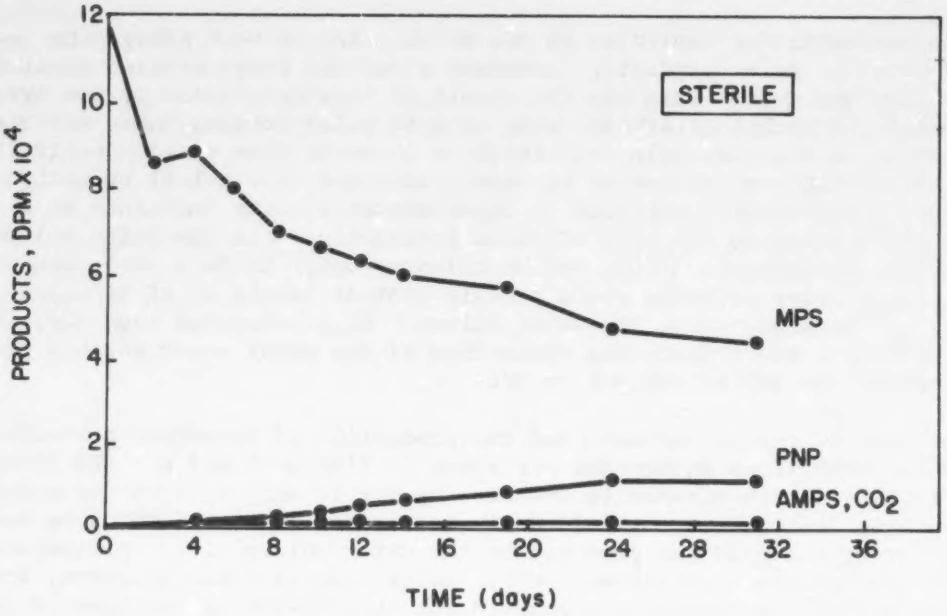

Figure 6. Products identified by thin-layer chromatography in methylene chlor-extractable material from water above the sediment in a sterile eco-core microcosm spiked with methyl parathion (0.5 mg/liter). MPS = methyl parathion, AMPS = aminomethyl parathion, PAP = *p*-aminophenol. Products are expressed as dpm/ml of water column. $^{14}CO_2$ is total produced averaged per days in collection period.

Only low levels of paranitrophenol (PNP) were produced in nonsterile cores; its absence may have reflected rapid metabolism or incorporation into microbial biomass. The production of PAP, in fact, could be from PNP rather than from AMPS. In other tests (see below), cores were shown to be capable of PNP metabolism after extended incubation periods.

PNP was the only product seen in sterile controls (the dimethoxy-thiophosphate hydrolysis product was not monitored in these studies), indicating significant hydrolysis of MPS by nonbiological processes. In all instances, the decrease of MPS in the sterile controls was accounted for by production of PNP. We have detected no enhanced hydrolysis due to the presence of formalin.

The production of ^{14}C-CO_2 (see Fig. 5) always commenced between days 4 and 10, peaking shortly thereafter. The initial lag may have resulted from a preferential metabolism of the acetone carrier. With continued incubation, production of CO_2 leveled off and eventually (day 28-32) began to decrease slowly. In most of the cores where methyl parathion was the carbon source, 10-15% of the parent compound was converted to CO_2.

Table 1 gives the material balance for an eco-core that incubated for 32 days. Most of the radioactivity in the sediments was associated with the detritus fraction and not with the sand fraction (low organic [1-2%], nonsuspendable particulate). In the nonsterile systems, 90% of the radioactivity associated with the detritus was tightly bound (not extractable with acetonitrile), and the remainder was loosely bound (extractable with acetonitrile). In sterile eco-cores the opposite was true: only a small amount of radioactivity was associated with the detritus fraction. Sand had little affinity for the radioactivity in either system. Overall, about the same total amount of radioactivity was found in the sediment, including interstitial water, regardless of whether it was sterile or nonsterile. However, in the sterile systems, 75% was found in the interstitial water; whereas, less than 40% was found in the interstitial water in nonsterile eco-cores. Degradation products found in interstitial water and associated with sediment (solvent extractable fraction) were the same as those found in the water overlying the sediment.

TABLE 1. MATERIAL BALANCE OF RADIOACTIVITY IN ECO-CORE WATER AND SEDIMENT[a]

Type of sample	Percentage of total ^{14}C			
	Sterile		Nonsterile	
Carbon dioxide	0.1		23.7	
Overlying water	62.2		41.3	
Extracted water[b]		20.1		28.4
Extractable products[b]				
MPS		62.7		3.9
PNP		16.3		2.2
AMPS		0.4		48.0
PAP		0.6		12.0
BASE		0.0		4.4
Detritus				
Extractable	6.0		1.6	
Bound	1.2		14.6	
Sand				
Extractable	1.0		0.4	
Bound	0.8		2.2	
Interstitial water	23.6		11.6	
Core wall washes	3.5		4.3	
Resin trap	0.0		0.0	
Percent recovery	90.1		92.4	

[a] Spiked with ^{14}C-MPS (0.5 mg/liter), analyzed after 32 d of incubation.

[b] Percentage figures are percent of total ^{14}C in overlying water.

EFFECT OF INTACT CORES

Several other experiments were carried out to determine the effects of microcosm design factors with the previous information as background for the typical fate of methyl parathion in these eco-cores. The necessity of using an intact sediment-water core was determined by assembling microcosms in two different ways: by the normal coring procedure (referred to as "intact" microcosms) or with a sediment-water mixture in the glass columns in which structuring occurred and allowed the mixture to settle out (referred to as "mixed"). Each set was spiked with ^{14}C MPS and the degradation processes monitored with time as described above.

Table 2 shows a partial summary of the results from the two types of eco-core microcosms. At the end of each incubation period (8 and 15 d) an entire microcosm was dismantled and analyzed. Activity was generally higher in the mixed systems. Total radioactivity in the water overlying the sediment was consistently lower in the mixed systems; this was complemented by increased levels of radioactivity bound to the sediments. Of the other compartments analyzed, only slight differences were noted; CO_2 production was also slightly higher in the mixed systems. Table 3 shows the results from product analyses of the mixed and intact eco-core experiments. No differences were observed in the types of products produced. Since CO_2 levels were lower in the intact core, a slower degradative process may be occurring resulting in the accumulation of intermediates.

TABLE 2. MATERIAL BALANCE OF RADIOACTIVITY IN ECO-CORE EXPERIMENTS USING MIXED AND INTACT SEDIMENTS

	Percentage of total $^{14}C^a$			
	Mixed		Intact	
	Day 8	Day 15	Day 8	Day 15
Water	57	49	74	63
Extracted water	6	2	5	5
CO_2	3	5	0.1	1
Detritus				
Extracted	4	2	5	1
Bound	36	39	15	25

[a] Total activity: 10 μCi (^{14}C) in 0.5 mg/liter MPS per core.

The differences of intact versus mixed eco-cores were determined further by monitoring CO_2 production from MPS over a 16-day incubation period when the water to sediment ratios (v/v) were varied. Results are shown in Figure 7. Mixed cores showed the greatest CO_2 production regardless of the water to sediment ratio. However, if water above the sediment (sediment volume maintained at approximately 50 ml) was reduced from 200 ml (5:1 water to sediment ratio) to 50 ml (1:1), CO_2 production decreased. Thus, water to sediment ratios affect CO_2 production and merit consideration in the design of microcosms.

During one of these experiments (see Fig. 7), ^{14}C ring-labelled *p*-nitrophenol (0.1 mg/liter) was added to the cores after 16 d of incubation with ^{14}C-MPS. An immediate increase in CO_2 production occurred, indicating that the microcosm system was acclimated to PNP metabolism while metabolizing MPS. Product analysis showed PNP disappearance was coupled with increases in polar degradation products (not shown). The mixed core systems again showed higher metabolic activity (CO_2 production) after PNP supplementation.

TABLE 3. PRODUCT ANALYSIS OF WATER OVERLYING SEDIMENTS (COLUMN) AND INTERSTITIAL WATER IN MIXED AND INTACT SEDIMENT OF AN ECO-CORE EXPERIMENT

Water	Days	Radioactivity (dpm x 10^3/ml)				
		MPS	PNP	AMPS	PAP	Base
Mixed						
Column	1	308	-[a]	-	-	-
	8	-	-	152	68	60
	15	1	2	-	11	9
Interstitial	1	13	2	-	35	16
	8	-	-	-	5	11
	15	4	1	-	6	7
Intact						
Column	1	242	23	-	41	51
	8	-	-	437	80	47
	15	15	20	19	91	131
Interstitial	1	24	81	-		-
	8	-	-	12	-	-
	15	3	1	1	4	6

[a] Missing values, radioactivity was not significantly above background.

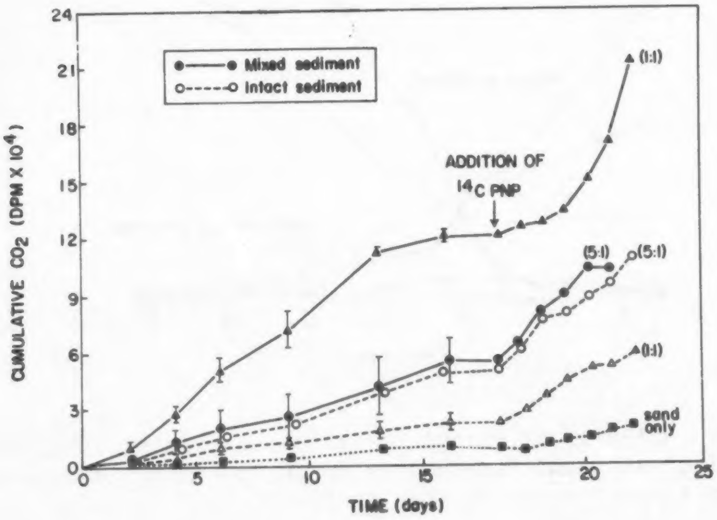

Figure 7. Cumulative $^{14}CO_2$ production from eco-core microcosms spiked with 0.5 mg/liter methyl parathion (MPS) as a function of sediment intact versus mixed, and as a function of sediment to water ratios (v/v), 1:1 and 5:1. *p*-Nitrophenol (^{14}C) was added at day 17 and $^{14}CO_2$ from both PNP and MPS was observed thereafter.

EFFECTS OF LABORATORY AGING

Aging or acclimation to laboratory conditions is an important consideration in microcosm experimentation. Accordingly, two types of intact eco-core systems were set up: one set was spiked with ^{14}C-MPS in previous experiments within 2 h after obtaining the fresh cores, the other set was spiked after the cores had aged (with continuous aeration) for 7 d in the laboratory at 25°C. Removal of MPS from the water above the sediment (Fig. 8) was slower in the aged microcosm, particularly over the first 4 days. Similar responses were apparent in the production of polar unextractable degradation products (not shown). Aminomethyl parathion and carbon dioxide production were also greater in the unaged or fresh system. In both instances (aged and unaged), degradation products, including CO_2, accounted for 85-95% of the loss of methyl parathion in the water, indicating that very little bound to sediments within the 16-d incubation period. The small amount of binding that did occur (5-10%) was the same regardless of aging. Overall, however, an unaged or fresh eco-core showed higher degradative activity.

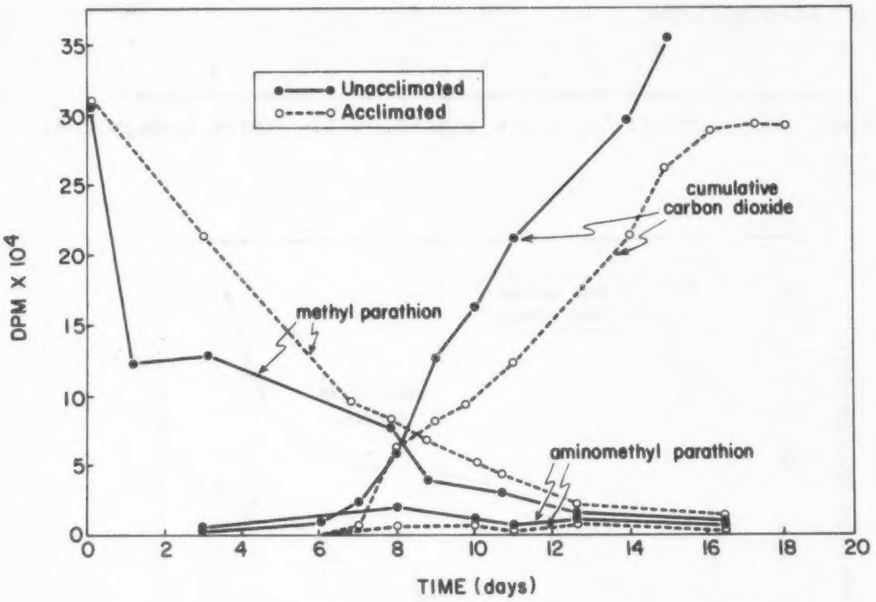

Figure 8. Changes in levels of methyl parathion, and aminomethyl parathion in overlying water and carbon dioxide in eco-core microcosms with and without laboratory aging (see text).

CONTINUOUS FLOW MICROCOSM

With these microcosms, the pesticide enters continuously. Since it takes time for the pesticide to diffuse into the sediment, a gradient will be established in the sediment. Consequently, the concentration of pesticide in the water overlying the sediment will increase with time, eventually equalling the influent concentration when equilibrium conditions result. In a sterile system where biologically mediated sorption to sediment or biodegradation will not occur, faster equilibrium should be attained. Changes in total radioactivity in water overlying sediment for sterile and nonsterile continuous flow microcosms are seen in Figure 9. Radioactivity in the water increased steadily in the sterile microcosm, eventually leveling off around 11.8 x 10^3 dpm/3 ml. Influent radioactivity was 12.1 x 10^3 cpm/3 ml, indicating the sterile control had reached equilibrium. In the nonsterile microcosm, the radioactivity in the water leveled off at values considerably lower than those observed in the sterile control. As demonstrated below, degradation contributed very little to loss of radioactivity from the water column. Thus, the leveling off of the radioactivity could be due to biologically mediated binding to sediment.

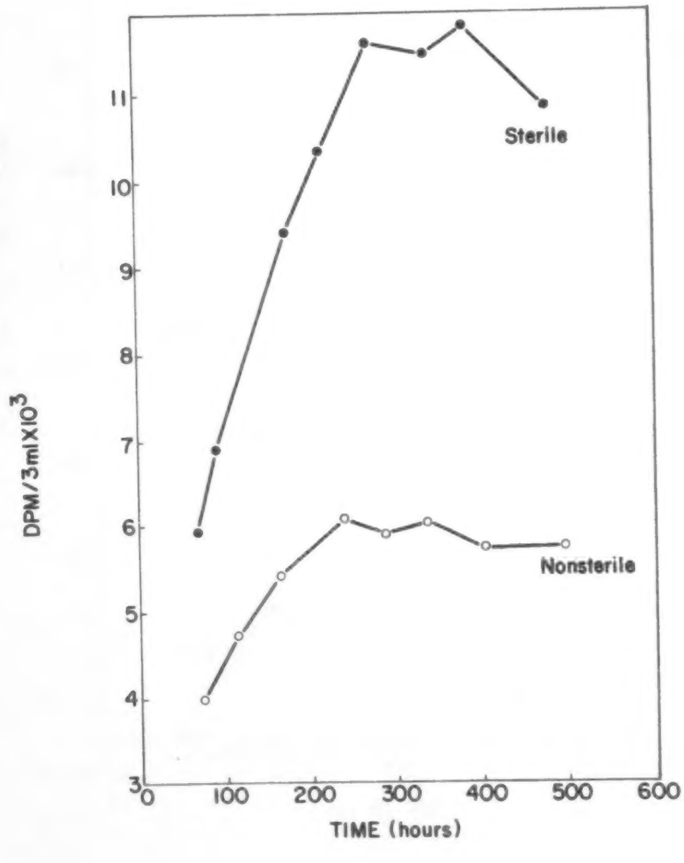

Figure 9. Total radioactivity in water above sediment in sterile and nonsterile continuous flow microcosm receiving continuous addition of ^{14}C-methyl parathion (50 μg/liter). Turnover time was 25 h.

Analysis of the degradation products generated in the water overlying the sediments is shown in Figure 10. In the sterile microcosm, the only degradation product was PNP. The decrease and eventual steady state in MPS concentration was compensated entirely by production of PNP. In nonsterile microcosms, the decrease in MPS concentration was greater and reached a much lower steady concentration level. The production of AMPS accounted for approximately 75% of the decrease in MPS; the remaining 25% was lost to polar degradation products and carbon dioxide. Only trace amounts of PNP were detected.

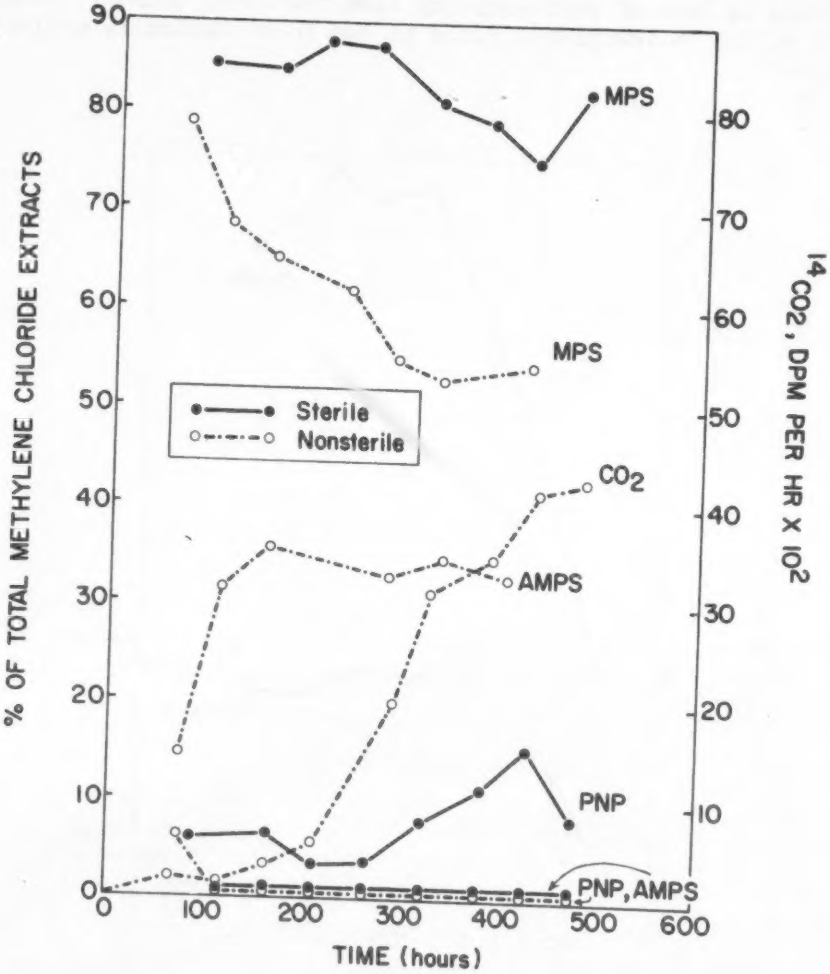

Figure 10. Products identified by thin-layer chromatography (% of total radioactivity) in methylene chloride extractable material from water above sediment in continuous flow microcosm receiving ^{14}C methyl parathion (50 µg/liter). MPS = methyl parathion, AMPS = aminomethyl parathion, and PNP = *p*-nitrophenol.

Table 4 shows the distribution of radioactivity in water and sediment of both sterile and nonsterile microcosms sampled at 425 h. Radioactivity, unextractable from water (polar degradation products), was always higher in nonsterile systems. Very little difference in total radioactivity was seen between water overlying the sediment and water within the sediment (interstitial). As seen with the eco-core microcosms, PNP was the only degradation product in the sterile controls. In nonsterile systems, no PNP was typically seen in interstitial water, perhaps signifying its metabolism to polar products (base), the latter also being higher in interstitial water. Higher levels of AMPS were also observed in nonsterile systems.

TABLE 4. MATERIAL BALANCE OF ^{14}C RADIOACTIVITY[a]

Substrate	Sterile	Nonsterile
Water		
Overlying (dpm/ml)	4,058 (19%)[b]	3,089 (31%)[b]
Interstitial (dpm/ml)	2,020 (4%)	3,725 (34%)
Detritus		
Extractable (dpm/g solids)	72,244	21,932
Bound (dpm/g solids)	14,700	87,693

Extractable products	Percentage distribution in water fractions			
	Overlying	Interstitial	Overlying	Interstitial
MPS	85	69	55	21
PNP	15	31	14	0
AMPS	0	0	16	46
PAP	0	0	10	0
BASE	0	0	5	32

[a] Water and sediment sampled at 425 h from continuous flow microcosm.

[b] Percent of ^{14}C unextractable from overlying water.

Large quantities of radioactivity were bound (not solvent extractable) to sediment material, particularly in nonsterile systems. The binding phenomenon was compound-dependent, since, in other studies with the same microcosms (Bourquin and Pritchard, unpublished), compounds like Kepone and diflubenzuron became bound to a much lesser extent.

ACCLIMATION TO PESTICIDE DEGRADATION

To determine the degree to which the systems acclimate to the degradation or transformation or a particular pollutant, two types of continuous flow microcosms were studied: one had a continuous exposure to unlabelled MPS (20 μg/liter) for 50 d, the other had an interval exposure to MPS of 25 d followed by a 25-d period of no exposure. On the 50th day, each system received ^{14}C

MPS continuously from a reservoir and the levels of total radioactivity in the water overlying the sediments and in the alkaline CO_2 traps were monitored. The results, as depicted in Figure 11, showed that the microcosms actually lost their original degradative capacity for MPS when not continuously exposed to the pesticide. Release of radiolabelled carbon dioxide in the acclimated system reached the same levels as observed in earlier experiments (Fig. 10). In the unacclimated system, however, the lack of exposure to methyl parathion resulted in lower levels of CO_2 production. The fact that CO_2 production remained in steady state indicated degradation capacity did not increase with time as might be expected.

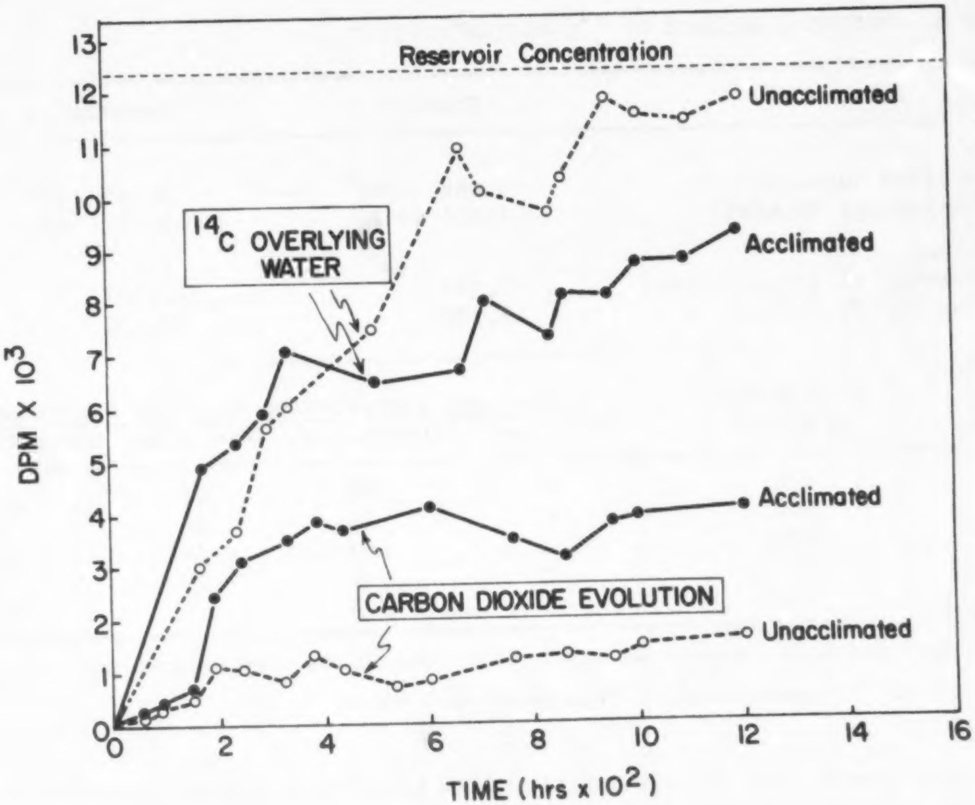

Figure 11. Total radioactivity in water (per 3 ml) above sediment and $^{14}CO_2$ production (per day) from acclimated (continuous exposure to methyl parathion for 50 h) and unacclimated (continuous exposure to methyl parathion for 25 h followed by no exposure for 25 h) and continuous flow microcosm receiving ^{14}C methyl parathion (20 μg/liter).

DISCUSSION

The application of microcosm techniques to studies of degradative processes in aquatic environments is relatively untested but holds promise as a potential laboratory tool to predict the rate and extents of fate processes in the real world (4,12,15,1). Use of radiolabelled materials and sterile controls further enhanced our evaluation of biological and nonbiological degradation processes; since aquatic environments are open systems with large dilution capacities, controlled field experiments, as executed in terrestrial environments (19), are prohibitive. Microcosms may be the next best mechanism for extracting information about fate and transport in aquatic habitats.

Microcosms also are an excellent method for assessing the interactive processes involved in fate and transport. Aquatic ecosystems are complex entities with many individual reactions dependent on output and input processes occurring in the surrounding milieu (8). We feel it is not prudent to base a study on fate of a chemical strictly on the results obtained from experiments on individual reactions examined apart from their more complex origin and chosen for study because of their amenability to defined laboratory experimentation. Such is often true with models developed to predict exposure concentrations in aquatic environments (6,2); individual processes like photolysis, hydrolysis, biodegradation are studied individually and then summed together to predict an overall effect. Laboratory isolation of individual components of a complex system often necessitates structuring the experiments so that the interpretation of results becomes meaningless. For example, descriptive analyses of biodegradation rates invariably fall back on Monod kinetics (3); this choice necessitates: a) the isolation of a pure culture that will grow on a pollutant as its sole source of carbon and energy; b) the determination of maximum growth rate (μ max) and substrate saturation constants (Ks), and c) the assumption that biodegradation is primarily a function of microbial growth under steady-state conditions. Each of these conditions is artificial to many "real-life" aquatic situations. For example, methyl parathion does not readily degrade in seawater in the absence of sediment (Pritchard and Bourquin, unpublished). When sediment is present, we have not been able to isolate a single organisms that was responsible for the degradation observed. Likewise, at very low substrate concentrations, degradation may be only indirectly related to microbial growth, making μ max and Ks determinations meaningless. Our studies, in fact, suggest that only small changes in microbial population density occurred in the continuous flow microcosms because acclimation did not occur in these systems (Fig. 11). Therefore, problems of interpretation arise when the experimental isolation of individual rate processes is required.

We envisaged a microcosm study that would monitor inputs and outputs rather than individual components; thus, interactive forces could be observed as a total effect. Our studies clearly showed that the rate and extent of formation of amino methyl parathion (AMPS), although documented in the literature (21,13,20,18,10), can probably be modeled in a microcosm study if reduced conditions in sediments can be regulated according to in-situ measurements. Naturally occurring sediments (range point) used in our studies typically have mild reducing conditions (Eh = +50 to -150) at the sediment-water interface before collections. Since these conditions are approximately duplicated in the microcosms and since we readily detect the production of AMPS in the microcosm, we would predict the production of AMPS under in-situ conditions. The formation of AMPS also represents a detoxification mechanism of MPS (16) and thus the effect on predicting exposure concentration is considerable.

Our microcosm studies also emphasize the need to more completely study the binding of MPS to aquatic sediments, particularly if sorption processes are to be modeled correctly for exposure concentration evaluation. It is apparent from our studies that the formation of bound residues (solvent unextractable) is greatly enhanced by biological activity, as exemplified by our use of sterilized microcosms. Katan et al. (13) and others have described similar binding phenomena for soils. The extent of this binding, its substrate specificity, and the environmental factors that control it, should be further studied. Data from our eco-core experiments (Fig. 5 and 6, Table 1) indicated that biologically mediated binding increased with continued incubation, i.e., loss of MPS from the water column initially was compensated for by the production of degradation products, not by binding to sediments. However, with continued incubation, more of the loss of MPS was accounted for by sediment-bound residues. Since an apparent acclimation was occurring, threshold concentration limits could exist in which minimal binding occurs if the MPS concentration in the sediment is below a certain level.

Katan and Lichtenstein (14) reported that the binding moiety of MPS was probably the reduced product, AMPS. Approximately half of the radiolabelled AMPS added to their loam soil became irreversibly bound within 24 h. Our results do not explicitly implicate AMPS as the bound component in aquatic sediments, but they do indicate that if AMPS is bound, it occurs at a much slower rate because it accumulates in the water phase (Fig. 5, 10). Overall, these results suggest that soil studies (sorption in particular) cannot be directly extrapolated to aquatic sediments.

Microcosms can be constructed in basically two ways: first by removing a portion of the environment with its complex components and studying them under controlled laboratory conditions or, second, by amassing together a limited number of individual organisms in some container in an attempt to model the complexity of a large ecosystem. We are of the impression that these latter types of microcosm studies are difficult to interpret, and susceptible to experimental bias (11,17) when applied to fate and transport studies. Microcosms which start with more complex components such as portions of a natural environment in a laboratory container are ultimately more amenable to studying fate processes and making predictions. These systems should have more semblance to real world situations because they potentially contain many of the interactive processes occurring in microenvironments. However, containerization processes may greatly affect validity of the data generated from any microcosm, especially when attempting to extract a simplistic answer from a complex system. Design features of a microcosm will also impact interpretation of the data generated therein. The size of the microcosm, water to sediment ratio, sediment surface area, sediment mixing, aeration, and nutrient regeneration are all critical factors which must be addressed before a microcosm can be successfully used and standardized.

Our data support the contention that design features will partly determine the final outcome of a fate study. With the eco-core microcosm, an intact sediment/water core had slower overall biological activity but larger amounts of degradation products than a mixed sediment/water sample. Actual intact cores probably give a more accurate assessment of the fate of MPS. These microcosm studies also reveal that degradation processes may be quite sensitive to perturbations in the sediments, such as natural dispersion and scouring brought on by different climatic regimes. A reduction of the water to sediment volume ratio also reduced degradation activity (as measured by CO_2 production) (Fig. 7). Aromatic ring cleavage mechanisms of *p*-nitrophenol, a precursor to CO_2 production, are probably restricted to aerobic habitats and unlikely to proceed in sediments (10). A reduction in water volume decreases the oxidizing zone and slows the microbial attack and eventual CO_2 release. Since natural estuarine environments are tidal, fluctuations in water level are typical, and degradation activities may vary accordingly.

Acclimation is another factor significantly affecting degradation activities. Laboratory acclimation of the eco-core microcosm, a closed system, had a depressive effect (significant changes in 7 d) on the processes contained therein. Within a closed system, only a finite concentration of microbial growth factors are available before regenerative processes supply these factors internally. We believe biodegradation studied during the regenerative phase (long periods of laboratory acclimation) will reflect potential characteristics of the laboratory system and not of the natural environment. With a closed system like the eco-core, our work indicates the most informative results are generated with as fresh a system as possible.

The continuous-flow microcosms demonstrated that acclimation to degradation of the pesticide did not increase after an initial incubation period (see Fig. 11), but in fact decreased if the pesticide was not continuously supplied at low levels. The results are not mutually exclusive regarding acclimation in the eco-core, but it is difficult to determine what actually happens in the natural environment. Since fresh nutrient, bacterial cells, and invertebrate animals continuously enter the microcosm (inflowing seawater), some unknown factor(s) is present in the original sediment that is not maintained unless methyl parathion is continuously supplied. We suspect this is related to a sediment-binding phenomenon because of the difference in equilibrium kinetics between an acclimated and unacclimated microcosm (see Fig. 11, radioactivity in water over sediment).

Additionally, we feel that carbon dioxide production considered alone in marine microcosms is a poor indicator of the extent of degradation (2% of total carbon added). These findings contrast sharply with many soil studies in which CO_2 may represent much more of the total test carbon added (19). In our studies with methyl parathion and other pesticides (unpublished), it was apparent that hydrolysis of the parent compound was far more indicative of the overall degradation process and that product analysis, other than CO_2, should be carried out in conjunction with a microcosm study.

The merits of an eco-core type microcosm relative to a continuous-flow microcosm are being defined and investigated. We developed a core-type microcosm as an aquatic equivalent to similar core-type microcosms developed for fate studies in terrestrial environments (7). These microcosms are simple, inexpensive, relatively rapid, and amenable to standardization for test protocols in registration programs. They are basically tier-two testing (tier-one being sewage sludge BOD test and/or batch enrichment studies), but they are limited to assessments of *degradation potential* only.

Continuous-flow microcosms are significantly more complicated but lend themselves to assessments of the *degradation capacity* of aquatic environments, i.e., the extent a particular aquatic environment will degrade or transform a pollutant and how fast these processes will occur. The virtue of assuming some sort of steady-state kinetics coupled with rate processes that are not limited by depletion of internal factors make continuous-flow microcosms potentially amenable to assessments of degradation potentials that truly reflect real life situations.

LITERATURE CITED

1. Ambrosi, D., A. R. Isensee, and J. A. Macchia. 1978. Distribution of oxadiazon and phosalone in an aquatic ecosystem. J. Agric. Food Chem. 26:50-53.

2. Baughman, G. L., and R. R. Lassiter. 1978. Prediction of environmental pollutant concentration, p. 35-54. *In* J. Cairns, K. L. Dickson, and A. W. Maki (eds.), Estimating the hazard of chemical substances to aquatic life. Amer. Soc. for Testing and Materials, STP 657, Philadelphia, Pa. 278 pp.

3. Bazin, M. J., P. T. Saunders, and J. I, Prosser. 1976. Models of microbial interactions in the soil, p. 463-498. *In* CRC Critical Reviews in Microbiology.

4. Bondietti, E. A., S. Draggan, J. R. Trabalka, and J. P. Witherspoon. 1974. State-of-the-art and recommended testing for environmental transport of toxic substances. U.S. Environmental Protection Agency, Environmental Sciences Division, Pub. No. 893. ORNL/EPA-1. 152 pp.

5. Bourquin, A. W., M. A. Hood, and R. L. Garnas. 1977. An artificial microbial ecosystem for determining effects and fate of toxicants in a salt-marsh environment. Dev. Ind. Microbiol. 18:185-191.

6. Branson, D. R. 1978. Predicting the fate of chemicals in the aquatic environment from laboratory data, p. 55-70. *In* J. Cairns, K. L. Dickson, and A. W. Maki (eds.), Estimating the hazard of chemical substances to aquatic life. Amer. Soc. for Testing and Materials, STP 657. Philadelphia, Pa. 278 pp.

7. Draggan, S. 1976. The microprobe analysis of ^{60}CO uptake in sand microcosms. Xenobiotica 6:557-563.

8. Floodgate, G. D. 1976. Decomposition processes in the sea with special reference to man-made wastes, p. 223-245. *In* 17th Symposium, The role of terrestrial and aquatic organisms in decomposition processes. Blackwell Scientific Publications, London.

9. Giddings, J. M., and G. K. Eddlemon. 1977. The effects of microcosm size and substrate type on aquatic microcosm behavior and arsenic transport. Arch. Environ. Contam. Toxicol. 6:491-505.

10. Graetz, D. A., G. Chesters, T. C. Daniel, L. W. Newland, and G. B. Lee. 1970. Parathion degradation in lake sediments. J. Water Pollut. Control Fed. 2:R76-R94.

11. Isensee, A. R., E. R. Holden, E. A. Woolson, and G. E. Jones. 1976. Soil persistence and aquatic bioaccumulation potential of hexachlorobenzene (HCB). J. Agric. Food Chem. 24:1210-1214.

12. Isensee, A. R., P. C. Kearney, E. A. Woolson, G. E. Jones, and V. P. Williams. 1973. Distribution of alkyl arsenicals in model ecosystem. Environ. Sci. Technol. 7:841-845.

13. Katan, J., T. W. Fuhremann, and E. P. Lichtenstein. 1976. Binding of ^{14}C parathion in soil: a reassessment of pesticide persistence. Science 193:891-894.

14. Katan, J., and E. P. Lichtenstein. 1977. Mechanism of production of soil-bound residues of ^{14}C parathion by microorganisms. J. Agric. Food Chem. 25:1404-1408.

15. Kearney, P. C., J. E. Oliver, C. S. Helling, A. R. Isensee, and A. Kontson. 1977. Distribution, movement, persistence, and metabolism of N-nitrosoatrazine in soils and a model aquatic ecosystem. J. Agric. Food Chem. 25:1177-1181.

16. Melnikov, N. N. 1971. Chemistry of pesticides. Springer-Verlag, New York.

17. Metcalf, R. L., G. K. Sangha, and I. P. Kapoor. 1971. Model ecosystem for the evaluation of pesticide biodegradability and ecological magnification. Environ. Sci. Technol. 5:709-713.

18. Munnecke, D. M., and D. Hsich. 1974. Microbial decontamination of parathion and p-nitrophenol in aqueous media. Appl. Microbiol. 28:212-217.

19. Sanborn, J. R., B. M. Francis, and R. L. Metcalf. 1977. Degradation of selected pesticides in soil: a review of the literature. U.S. Environmental Protection Agency Ecological Research Series, EPA-600/9-77-022, Cincinnati, Ohio. 615 pp.

20. Schulz, K. R., and E. P. Lichtenstein. 1971. Field studies on the persistence and movement of dyfonate in soil. J. Econ. Entomol. 64:283-286.

21. Sethunathan, N. 1973. Microbial degradation of insecticides in flooded soil and in anaerobic cultures. Residue Rev. 47:143-165.

22. Taub, F. B. 1969. A continuous gnotobiotic ecosystem, p. 101-120. *In* The structure and function of fresh-water microbial communities. Res. Monogr. 3, Virginia Polytechnic Inst., Blacksburg, Va.

BIODEGRADATION IN MICROCOSMS: APPLICATION OF MATHEMATICAL MODELING

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ABSTRACT

The use of mathematical modeling as a tool to study biodegradation in natural environments is discussed. Both suitability and limitations of the approach are considered in the light of other biological systems. The usefulness of simulation in hypothesis testing is illustrated by manipulating the microbial component of a lake ecosystem model. The effects of the removal of microbial mineral mobilization, phytoplankton productivity and allochthonous organic loadings on organic matter and nutrient pools are demonstrated.

Perhaps the nature of ecology is the best argument for the role of systems theory in ecological inquiry. Biology has been defined by Weaver (5) as "organized complexity" among the natural sciences. This is never seen so clearly as in ecosystem studies. Some general statements can be made about ecosystems (and microcosms) that indicate their suitability for the methodology of mathematical modeling: a) they are complex and multivariable; b) many functional components are imperfectly measured or even unidentified; and c) multilevel activities are strongly interconnected functioning to maintain a reasonably steady state overall. Thus, these systems have properties similar to biochemical and physiological systems and as such are amenable to application of organized analytical and modeling techniques.

An example of the modeling of a biochemical system is Hess' model (4) of glycolysis. More than 89 reactions among 65 chemicals are described in this model, and good agreement with experimental data, i.e., flux, concentration of intermediates, etc., is observed; however, it took half a century of biochemical investigation to gather the data for this feat.

In ecosystem studies, there is an *additional* constraint to applying mathematical models and in particular computer program models. This is the innate uncertainty that the data are reproducible, and that what has been measured is *representative* of the system. Yet ecosystems are not the only systems that suffer from variability. Toxicity studies are done with hundreds of mice for statistical evaluation of the data, since great differences in susceptibility are seen in laboratory mice.

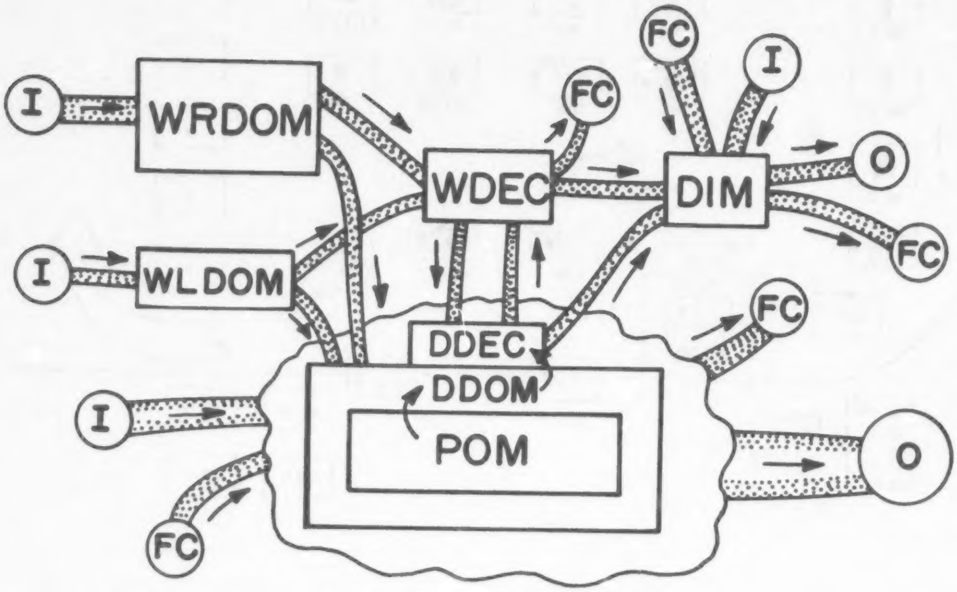
In our example of glycolysis, we assume that the homeostatic apparatus of the organism will maintain the temperature, the hydrogen ion concentration, ion balance, glucose pool, etc., such that the cellular environment is reasonably constant. Within reason, the processes of natural selection and adaptation produce the same kind of stability in the environmental system. However, this means that our environmental system is changing, perhaps not as much in function as in structure. In the first instance, the environment is kept "constant" by the activities of cells and in the second, the environment forces adaptation or selection to the system.

This, of course, is the basis for concern, since much of the experimental data used in model construction have been gathered in laboratory investigations of environmental samples. One can assume that community structure of water or sediment is determined by certain major environmental variables, and that given those variables, a certain community structure will result (subject to change by the laboratory environment). This laboratory effect is probably no worse than comparing mice to men in toxicity studies. Laboratory data, however, are influenced by seasonality of the sample and population shifts from changing growth conditions upon bringing the sample into the laboratory. While laboratory data are valuable, it may be different from that obtained in the field. This is no doubt similar to differences in biochemical activities of intact and excised tissue, yet most biochemical studies are done with excised tissue.

Testing certain hypotheses derived from the laboratory, or performing certain experiments in ecosystem studies may be virtually impossible. One cannot sacrifice an ecosystem very readily. There are examples of such large-scale experimentation (e.g., Hubbard Brook), but for most needs these systems are not available. This use, simulation, is a unique application of computer programmed models.

We have simulated several conditions that could not be experimentally tested to probe the sensitivity of a general lake ecosystem model to various perturbations (1). The microbiological detail of the lake ecosystem model CLEANER, illustrated in Figure 1, shows the various compartments of the microbial growth and decomposition components of CLEANER. Figure 2 shows the relationship of the microbial component to the rest of the compartments in the lake ecosystem model.

Using a computer programmed mathematical model developed for Lake George in upstate New York as part of the International Biological Program, we determined the differential rates of uptake by the microflora during the course of a year as modified by environmental variations. As shown in Figure 3, the model predicts dynamic changes in uptake rates as a result of inputs from spring runoff and spring primary productivity. The evidence that spring runoff is an important input to the lake is given in Figure 4. One of the major tributaries to Lake George, Northwest Bay Brook, drains a large percentage of



FC - CONNECTION WITH FOOD CHAIN.
I - INPUTS FROM LAND, ATMOSPHERE, LITTORAL ZONE AND BOTTOM SEDIMENT.
O - LOSSES TO ATMOSPHERE AND BOTTOM SEDIMENT

Figure 1. Compartmental diagram of microbial growth and decomposition model.
WRDOM, Refractory dissolved organic matter in water
WLDOM, Labile dissolved organic matter in water
WDEC, Decomposing microorganisms freely suspended in water
DDEC, Decomposing microorganisms attached to particulate organic matter
DDOM, Dissolved organic matter sorbed to particulate organic matter
POM, Particular organic matter
DIM, Dissolved inorganic matter

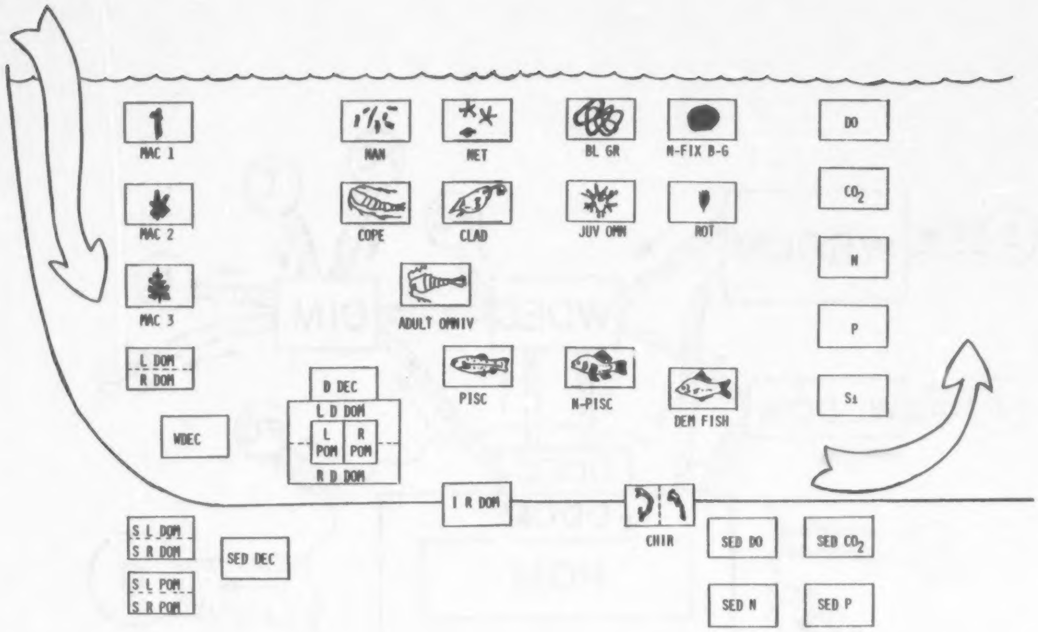


Figure 2. Compartmental diagram of lake ecosystem model (3).

MAC 1,2,3, Three macrophytes of differing growth habits
NAN, Nannophytoplankton
NET, Net phytoplankton
BLGR, Non-N_2 fixing blue-green algae
N-FIXBG, N_2-fixing blue-green algae
COPE, Copepods
CLAD, Cladocerans
JUVOMN, Juvenile omnivorous zooplankton
ROT, Rotifers
ADULTOMN, Adult omnivorous zooplankton
PISC, Piscivorous fish
N-PISC, Non-piscivorous fish
DEMFISH, Demersal fish
LDOM, Labile dissolved organic matter
RDOM, Refractory dissolved organic matter
WDEC, Decomposing microorganisms freely suspended in water
DDEC, Decomposing microorganisms attached to particulate organic matter
LDDOM, Labile dissolved organic matter sorbed to particulate organic matter
LPOM, Labile particulate organic matter
RPOM, Refractory particulate organic matter
RDDOM, Refractory dissolved organic matter sorbed to particulate organic matter
IRDOM, Interstitial water refractory dissolved organic matter
CHIR, Chironimids
SLDOM, Sediment labile dissolved organic matter
SRDOM, Sediment refractory dissolved organic matter
SLPOM, Sediment labile particulate organic matter
SRPOM, Sediment refractory particulate organic matter
SEDDEC, Sediment decomposing microorganisms
DO, Dissolved oxygen
CO_2, Carbon dioxide
N, Nitrogen compounds in water
P, Phosphorus compounds in water
S, Sulphur compounds in water
SEDDO, Sediment dissolved oxygen
SEDN, Sediment nitrogen
$SEDCO_2$, Sediment carbon dioxide
SEDP, Sediment phosphorus

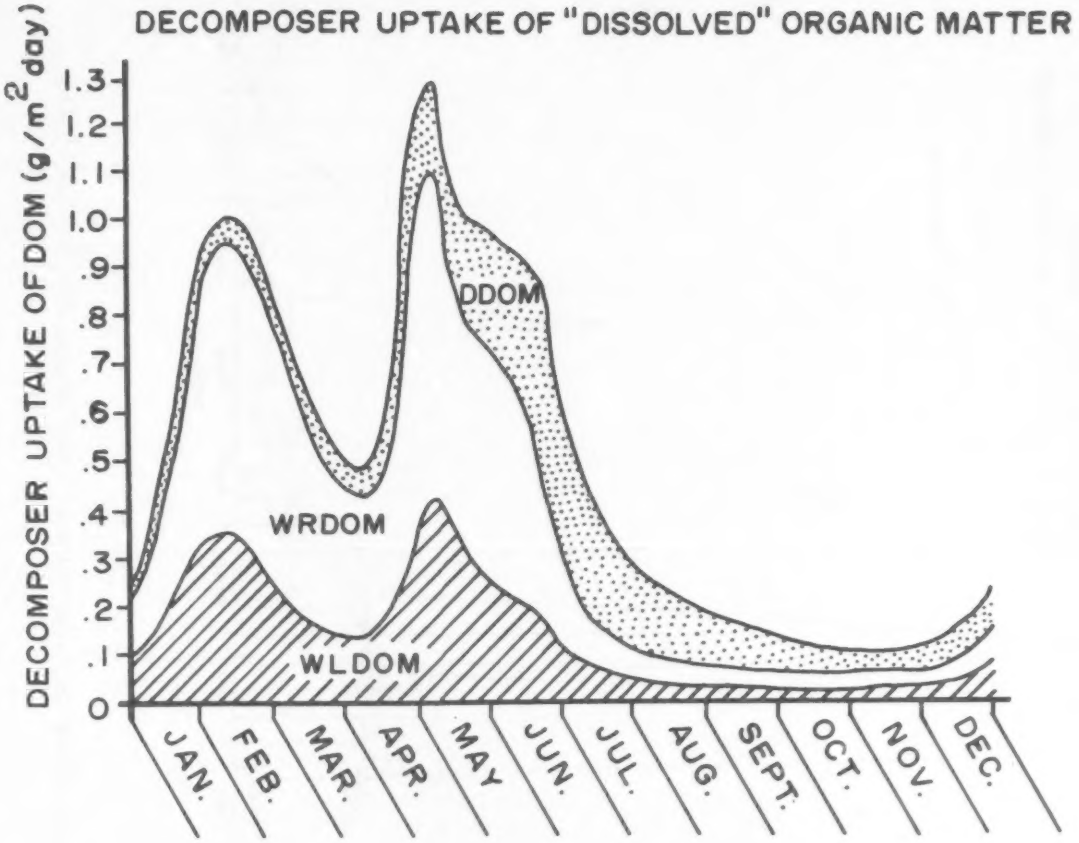


Figure 3. Decomposer uptake of dissolved organic matter.

DDOM, Dissolved organic matter sorbed to particulate organic matter
WRDOM, Refractory dissolved organic matter in water
WLDOM, Labile dissolved organic matter in water

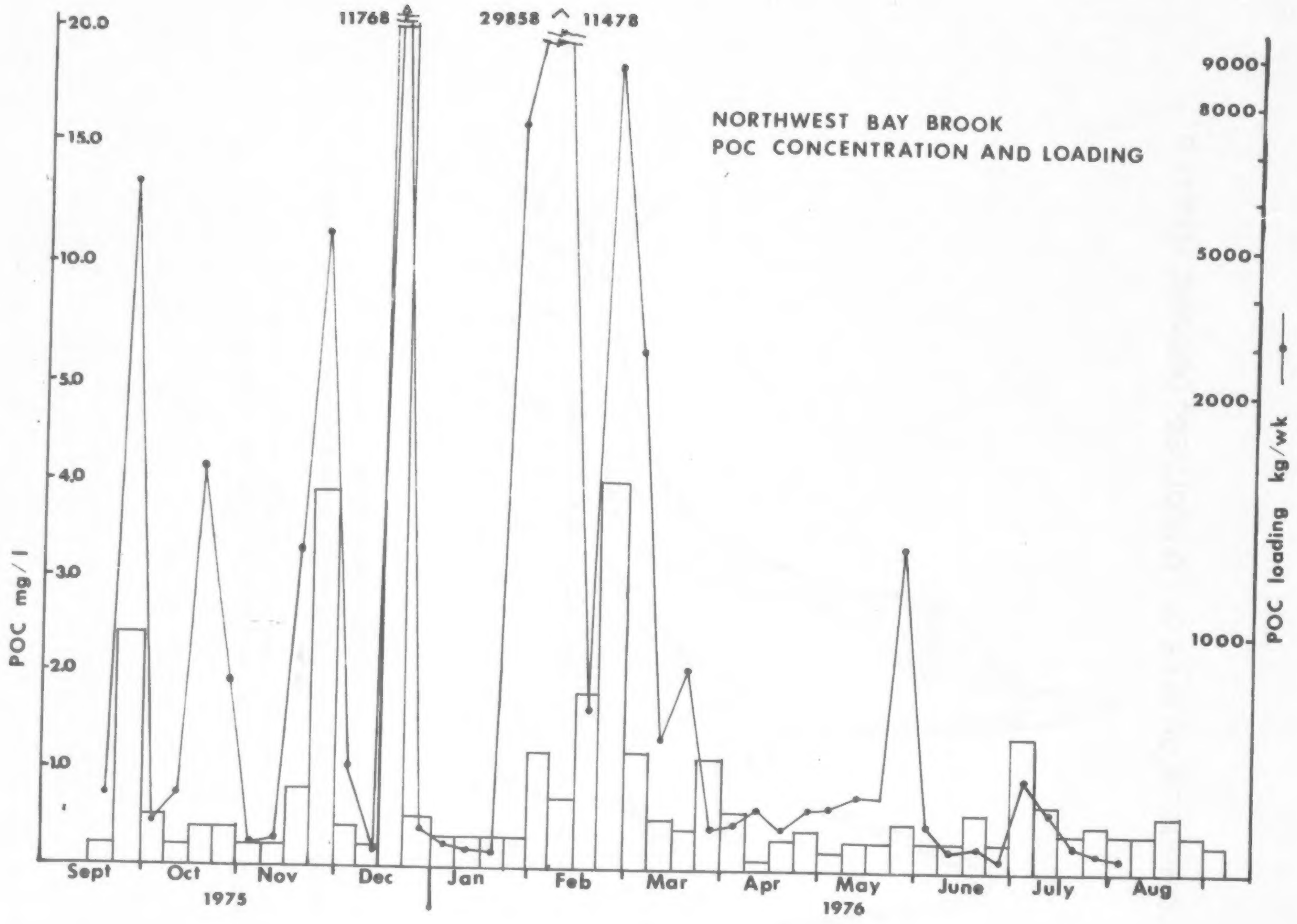


Figure 4. Northwest bay brook particulate organic carbon (POC) concentration and loading.

the basin, and as such is an important source of allochthonous material to the lake. We have been able to validate these uptake rates for DOM using growth rate data from continuous culture and isotope uptake experiments (2).

The sensitivity of the model to changes to conditions that could not be studied experimentally are illustrated in Figures 5 and 6. Figure 5 shows the response of the decomposer and organic matter compartments to normal and perturbed conditions in Lake George, New York, over a period of 827 d. In the normal simulation (Fig. 5A), the annual cycles are evident, and the model is seen to be stable with a large, but realistic, fluctuation in the freely suspended decomposers.

When the decomposers are removed from the system, the response is significantly different (Fig. 5B). Labile DOM increases rapidly, while refractory DOM increases more slowly. POM exhibits a stable seasonal fluctuation but, as will be shown later, does not reach the peak concentrations that are attained with normal seasonal contributions from algal production. In the absence of heterotrophic uptake, sorbed DOM exhibits slight annual peaks that do not occur under normal conditions.

A simulation without influence of phytoplankton is shown in Figure 5C. In this detritus-based system, the decomposer and organic matter compartments maintain stable, annual patterns of oscillations. However, the patterns reflect the seasonal loadings of organic matter. Without autotrophic production in the late spring and summer, the freely suspended decomposers, and to a lesser extent the POM and attached decomposers, exhibit pronounced troughs. The abnormally low microbial activity, in turn, results in a slight seasonal rise in refractory DOM that is not seen in a normal simulation.

The complete absence of allochthonous organic loadings (Fig. 5D) leads to an entirely different series of patterns of declining concentration. This suggests that with the present calibration the decomposition submodel is overly sensitive to external loadings. This sensitivity probably reflects our inadequate linkage between littoral and pelagic zones. Interestingly, the DOM compartments show little change; they are receiving some input from autochthonous sources but are not subject to the solubilization and sedimentation that affect the POM pool.

Figure 6 demonstrates the control that remineralization and autotrophic uptake exercise over the nutrient pools. In Lake George the dissolved oxygen and CO_2 levels are rather uninteresting. However, in a normal simulation (Fig. 6A), phosphate exhibits the effects of remineralization followed by severe depletion as a result of phytoplankton growth. Nitrogen has a less marked seasonal fluctuation.

Figure 6B indicates that there is no remineralization by decomposers, and phosphate disappears as soon as the growing season begins. Soluble inorganic nitrogen is remineralized by higher organisms and also is brought in through precipitation and runoff so that it gradually increases in concentration.

In the absence of uptake by phytoplankton (Fig. 6C), nitrogen, phosphorus, and carbon increase in concentration. Without organic loadings (Fig. 6D),

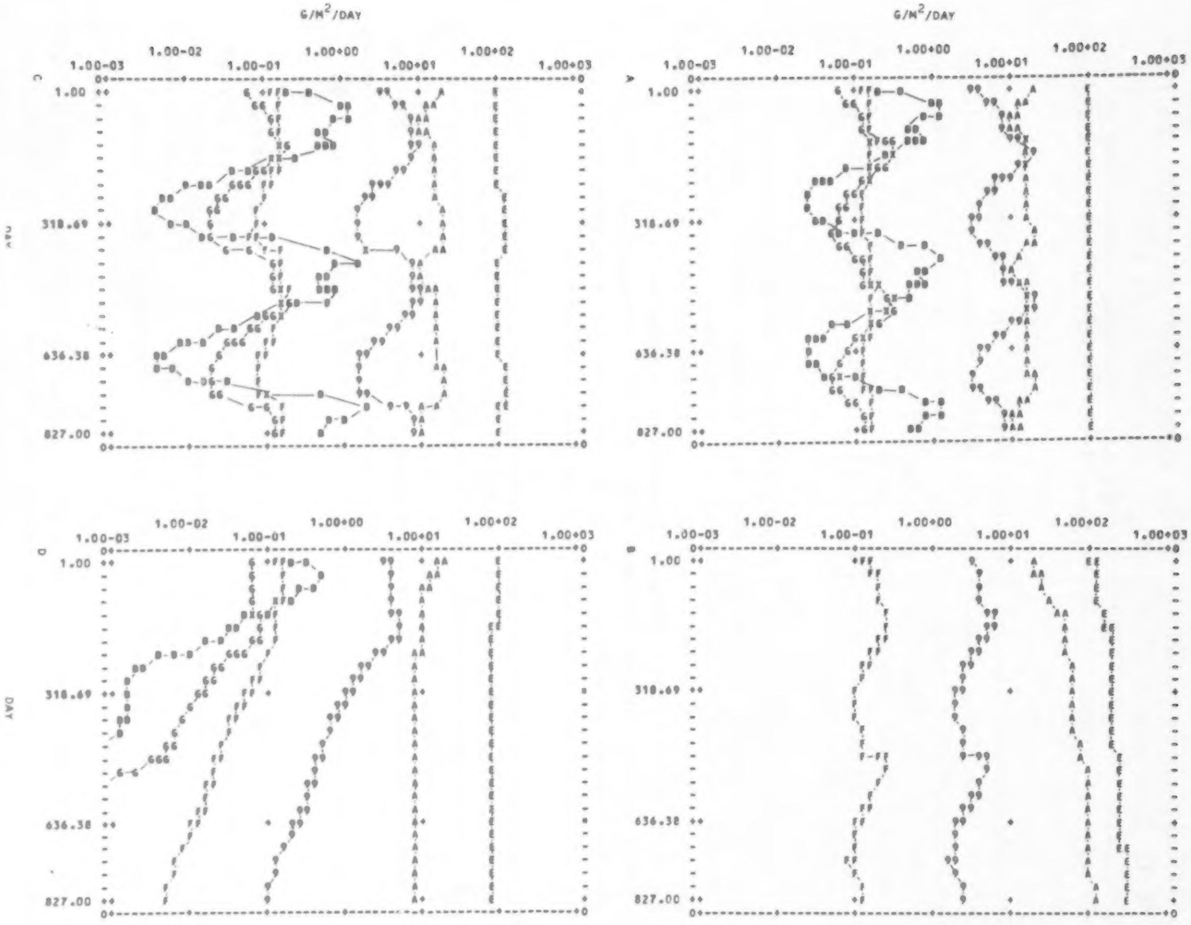


Figure 5. Example of a simulation. The response of decomposer and organic matter compartments are shown under (A) control conditions, (B) in the absence of decomposers, (C) in the absence of phytoplankton, (D) in the absence of allochthonous organic loading. Symbols: A, WLDOM; B, WDEC; E, WRDOM; F, DDOM; G, DDEC; 9, POM.

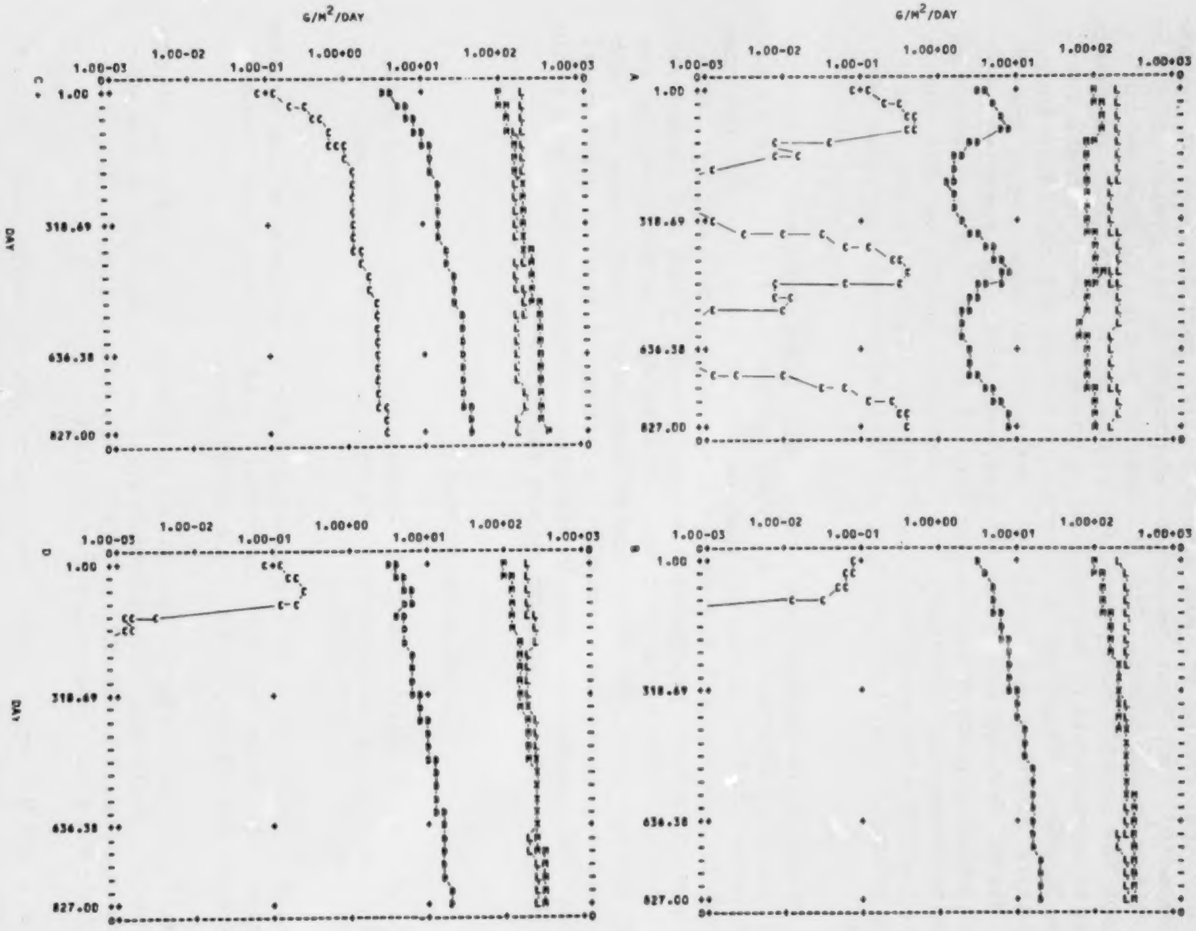


Figure 6. Example of a simulation. The response of nutrient pools are shown under (A) control conditions, (B) in the absence of decomposers, (C) in the absence of phytoplankton, (D) in the absence of allochthonous organic loading. Symbols: C, soluble reactive phosphate; D, soluble inorganic nitrogen; L, dissolved oxygen; M, carbon dioxide.

there is an initial mobilization of phosphate by decomposers; otherwise, the effects of decomposition are limited, and the result is similar to that of no decomposers (Fig. 6B).

How does this exercise help us understand actual situations in aquatic ecosystems? It should be possible to classify aquatic systems according to a group of key functional variables, and within a class certain types of responses should be expected, such that responses determined in one system should be applicable to others in the class. The problem, of course, is that we don't know enough about how aquatic ecosystems are regulated to have this confidence. But the problem of environmental degradation is prevalent and the means for treating it are limited. Therefore we have to implement our available tools with the recognition of their shortcomings. Mathematical modeling is one of those tools. Careful development, constant testing, continual refining, and cautious use of computer program models can lead to information on biodegradation in aquatic environments. However, the information cannot be more than hypothetical in perturbation studies and predictions. Nevertheless, a good hypothesis is a valuable step in scientific investigation and the basis for many a decision.

The tight coupling of mathematical models with laboratory and field investigations is the best approach when applying models for the purpose of deriving information related to biodegradation in the environment. The enormous complexity of the system suggests the use of a systematic methodology. However, modeling should not be considered a substitute for field and laboratory investigation. Rather, models are used most beneficially in facilitating the organization of diverse data, the detection of conceptual weaknesses, and the testing of hypotheses that may be virtually impossible to test in other ways. With the same kind of reservation used in applying cellular studies to a whole organism, models can be used to predict system responses.

LITERATURE CITED

1. Clesceri, L. S., R. A. Park, and J. A. Bloomfield. 1977. General model of microbial growth and decomposition in aquatic ecosystems. Appl. Environ. Microbiol. 33:1047-1058.

2. Clesceri, L. S. 1972. Role of the heterotrophic microflora in the cycling of materials. Oak Ridge National Laboratory, Eastern Deciduous Forest Biome--IBP Memo Report No. 72-64. Oak Ridge, Tenn.

3. deCaprariis, P., R. A. Park, R. Haimes, J. Albanese, C. Collins, C. Desormeau, T. Groden, D. Leung, and B. Youngberg. 1977. Utility of the complex ecosystem model MS.CLEANER, p. 87-89. *In* Proceedings of the International Conference on Cybernetics and Society.

4. Garfinkel, D., and B. Hess. 1964. Metabolic control mechanisms. VII. A detailed computer model of the glycolytic pathway in *Ascites* cells. J. Biol. Chem. 239:971-983.

5. Weaver, W. 1948. Science and complexity. Am. Scientist 36:536-544.

MICROBIAL COMMUNITY STRUCTURE

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ABSTRACT

It is generally recognized that most natural environments contain a heterogeneous assemblage of microbes. In some instances this has resulted in evolution of mutually beneficial associations of different microorganisms, although the extent of their occurrence, distribution and general significance remains to be fully assessed. Microbial communities show varying levels of structural complexity and degrees of interdependence between the component populations. They may be based on non-specific interactions which result in loosely structured communities with variable component populations. In contrast, microbial communities can be tight associations based upon a specific, perhaps obligatory, interaction.

Mixed cultures may have a crucial role in biodegradation of complex natural products or xenobiotics. In some instances it is difficult to isolate a single species with the complete capability to metabolize a given complex substrate. Nevertheless, such degradation does occur in nature at a reasonable rate and this may be the result of the combined activities of several organisms with separate, but complementary, degradative capabilities. Similarly, the breakdown of a xenobiotic could involve several steps catalyzed by enzymes produced by different microbes or the evolution of novel or improved catalytic activites in an existing enzyme system. In either situation, multispecies communities provide a stable, "permissive" environment for development of the requisite capability. One important aspect may be cometabolism where products which cannot be further metabolized in a pure culture can be used by other organisms in a mixed culture. Furthermore, the significance of the role of degradative plasmids may be enhanced within a microbial community since there is a greater genetic potential.

Traditional enrichment and isolation techniques favor pure cultures, whereas continuous-flow culture techniques can be used to isolate interacting associations of microorganisms. The advantages and disadvantages of this technique in examining adaptive processes in mixed cultures is discussed.

INTRODUCTION

Although it is generally accepted that most natural environments contain heterogeneous assemblages of microorganisms (4), little is known about the consequences of growth and coexistence of different microbial populations in the same niche. It may be that much of the activity of different populations is comparatively independent, in which instance the traditional methods of isolating and examining pure cultures should provide the necessary information on the role of that population in nature. However, it seems improbable that, during the course of evolution, interactions and relationships between different populations have not developed. Indeed, it is now becoming clear that microbial communities, i.e., mutually beneficial units composed of different species of microorganism, do occur in nature; these may be isolated if appropriate enrichment and selection techniques are employed. Perhaps because of the emphasis upon the isolation of single species of microbe, relatively few examples of structured microbial communities have been described. Certainly, the extent of their occurrence, distribution and overall significance in natural habitats remains to be fully assessed. Thus, scant attention has been paid to the physiological, biochemical and genetic consequences of interactions between different populations.

This communication describes some of the types of microbial communities that have been isolated and characterized and discusses the use of continuous-flow culture techniques in isolation of stable mixed cultures. In addition, the relevance of the growth of mixed cultures with respect to biodegradation of environmentally undesirable compounds and the evolutionary potential of microorganisms within a community structure are discussed.

THE STRUCTURE OF MICROBIAL COMMUNITIES

Although a number of basic types of interaction between different microbial populations have been recognized (28, 35, 36), it is at present difficult to assert that all the potential categories of microbial community have been observed. A basic feature, however, of microbial communities is that they constitute a group of microorganisms which grow together in the same environment. In many instances, it is also possible to recognize a mutual benefit between the component populations. Thus, microbial communities show a wide range of structural complexity and degrees of interdependence between the component populations. On the one hand they may be based on non-specific interactions which result in formation of loosely structured communiities. Indeed, it may be argued that some of these loose associations ought not to be considered as authentic communities. For example, there is clearly some interaction between the various populations involved in successions or particular sequences in biogeochemical cycles (i.e., nitrification) where the growth of one population sufficiently alters the environmental conditions permitting the growth of a second, succeeding population. In most situations, there is a characteristic sequence of population changes, temporally separated, but obviously comprising an interacting group of organisms. Furthermore, under appropriate culture conditions it is possible to maintain a stable mixed culture of part or the whole of the succession or cycle. It can be argued that from such a loose association of different populations potentially important specific communities may be evolved under the appropriate environmental conditions.

Alternatively, microbial communities can exist that are based on specific, obligatory interactions such that none of the component populations are able to grow alone. It is these categories of microbial communities which are likely to be overlooked since conventional enrichment and selection procedures favor the isolation of single species able to grow in a particular environment. A number of different categories of tight microbial communities have been recognized, at least for the simpler, smaller communities (35).

Microbial communities may be based on reciprocal, specific nutritional interactions between the component populations. In the simplest example, namely a two-membered community, two auxotrophic populations can have their requirements met in part by the other population. For example, Nurmikko (31) isolated a community composed of a phenylalanine-requiring strain of *Lactobacillus plantarum* and a folic acid-requiring strain of *Streptococcus faecalis*. The two populations could be grown together, but not separately, in a medium lacking both growth requirements since the *Lactobacillus* excreted folic acid and the *Streptococcus* provided phenylalanine. Among a number of similar communities (3, 10, 23, 26, 39, 42), one of the more recent has been the characterization of a simple two-membered community capable of utilizing cyclohexane as the sole carbon and energy source (37). Considerable difficulties have been experience in the past in attempting to isolate a pure culture able to use cyclohexane, although the compound has been known to be readily degraded in the natural environment. This particular community was isolated from an estuarine mudflat and the primary organism capable of metabolizing cyclohexane was identified as a species of *Nocardia*. However, the primary organism grew only in the presence of a pseudomonad which was subsequently shown to be supplying the actinomycete with biotin. In turn, the pseudomonad was supplied with metabolizable carbon and energy sources derived from either the immediate degradation of cyclohexane or cell lysis products.

More complex interactions may occur leading to the formation of closely coupled communities. In 1943, Schopfer (33) described an association of a species of *Mucor* and *Rhodotorula*. Both organisms required the vitamin thiamine but separately each organisms was unable to synthesize the compound. Thiamine has two major precursors, namely pyrimidine and thiazole, and it was shown that the *Mucor* species could only synthesize the former component while the *Rhodotorula* species produced the latter. Thus, as a community these two organisms were able to complete the synthesis of the common requirement for growth. More recently, Bates and Liu (2) found that a mixed culture of two pseudomonads resulted in production of lecithinase, whereas separately neither culture contained this activity. The implication is that the two strains produced different enzyme subunits which together resulted in the formation of a fully functional enzyme.

The ubiquity or otherwise of these specific kinds of association in natural habitats has not been examined in any detail, although it is our view that they are likely to be much more common than has so far been appreciated. First, there is considerable circumstantial evidence that specific nutritionally based associations do occur. For example, many marine bacteria have been observed to grow in close proximity to algae and the heterotrophs are known to excrete vitamin B_{12} which may enhance the growth of the algae (4). Moreover, it is probable that the bacterial populations derive some benefit from the association through the excreted reduced carbon compounds and oxygen produced as a result of algal photosynthesis. It would be extremely interesting to know how significant these relationships are in promoting the growth of the mixed cultures compared with growth as individual populations.

Second, specific associations are likely to be overlooked because of the selection and isolation procedures used to examine the microbial flora of a particular habitat. Conventional techniques result in the disruption of population interactions leading to lack of growth of the component populations during the selection procedures.

Stable associations can be based upon the removal of a growth inhibitory compound which is either produced by one of the members of the community or may be present in the growth environment initially. For example, an organism originally known as *Chloropseudomonas ethylica*, a photosynthetic bacterium with the unusual capacity to utilize ethanol as a photosynthetic electron donor, has now been shown to be a tightly coupled microbial community composed of a photosynthetic green sulphur bacterium and a sulphate-reducing bacterium (13). The most important interaction, although there may be others concerned with the carbon nutrition of the two organisms, regarding the utilization of oxidized and reduced sulphur compounds. The photosynthetic organism required hydrogen sulphide as an electron donor, resulting in its oxidation to sulphate, while the sulphate-reducing bacterium required sulphate as a terminal electron acceptor, regenerating hydrogen sulphide. Thus, by growing as a community the two organisms were able to recycle limited quantities of sulphur, thereby sustaining growth of both populations. The importance of this relationship to both organisms is that neither has to grow in a high sulphide-containing environment which is toxic to both populations. A number of other less obligatory relationships fulfilling the same metabolic function have been identified (40).

Wilkinson et al. (38) have described another microbial community containing at least four different species and capable of growing on methane as the sole carbon and energy source. Only one member of the community, an unidentified pseudomonad, was able to oxidize methane which resulted in some excretion of methanol when the organism was grown in pure culture. Methanol accumulation resulted in inhibition of the pseudomonad. However, within the community, a species of *Hyphomicrobium* oxidized the available methanol as its primary carbon and energy source, thereby preventing the accumulation of methanol to toxic levels and so maintaining the growth of the methane-oxidizing pseudomonad. Together these two populations comprised greater than 95% of the total community under all the growth conditions examined. However, it is uncertain what function the two minor populations, a *Flavobacterium* sp. and an *Acinetobacter* sp., had in the structure of the community. It was postulated that these species grew on cell lysis products and their presence ensured the maximum utilization of all the available carbon and energy sources. If this is true, then the presence of these two populations may confer little direct advantage to the major groups and simply be retained within the growth environment because they occupy an available growth niche (16).

A number of different mixed cultures have been described, particularly for rumen microorganisms, which depend on the transfer of hydrogen ions (or electrons) between the component populations. For example, the organism known as *Methanobacillus omelianskii* was found to be a tight association of two bacteria with the overall capability of utilizing ethanol as a carbon and energy source, resulting in the production of methane (6). One component of the community was termed the S organism and oxidized ethanol to acetate with the concomitant production of hydrogen. In pure culture the presence of hydrogen caused the cessation of S organism growth, whereas in the mixed culture, growth continued because the hydrogen ions were used as an energy source by the second component, namely *Methanobacterium* strain MOH. The methanogenic bacterium used carbon dioxide as an electron acceptor producing methane as a final product. The specific association of these two organisms maximized the amount of new biomass produced from the oxidation of unit amount of ethanol and, as with "*Chloropseudomonas ethylica*," ensured the immediate removal of an inhibitory waste product in order to sustain growth of the hydrogen-producing organism. The association of organisms in *Methanobacillus omelianskii* was not obligatory since other methanogenic bacteria, for example *Methanobacterium ruminantium*, could grow in association with the S organism. Subsequently, a number of other communities, in which excess reducing power produced by one population is immediately used by a second, have been isolated (5, 9, 20).

Insufficient attention has been given to the possibility that associations, particularly those based on loosely grouped organisms, may occur on the basis of overall basic growth characteristics determined by the two fundamental growth parameters, namely maximum specific growth rate and the growth-limiting substrate saturation constant. The maximum specific growth rate of a group of organisms may be greater than the individual growth rates of the separate populations grown under the same conditions. Osman et al. (32) isolated a stable, three-membered mixed culture using a chemostat continuous-flow culture system with orcinol as the sole carbon source. A single primary population, *Pseudomonas stutzeri*, metabolized orcinol and grew in pure culture on this substrate and in the mixed culture was the dominant population. The two secondary populations, unable to grow on orcinol, comprised up to 20% of the total biota depending on the growth conditions. Acetate was identified as a major metabolite excreted by the primary population which, together with cell lysis products, supported the growth of the secondary populations. A significant point was that *P. stutzeri* was capable of good growth on orcinol in pure culture indicating that there was little direct or obligatory interaction with the two secondary populations. Two possible reasons may explain the continuing presence of the secondary populations and the extreme stability of the three-membered mixed culture which was continuously cultured for several thousand hours during which period it was subjected to a number of environmental stresses, including temperature changes and alterations in the concentration of the growth-limiting substrate. First, the association could have been the result of a fortuitous presence of the secondary populations. If the concentrations of growth substrates required to support the growth of the secondary populations were high enough to sustain growth rates which were greater than the specific growth rate of the primary population, then the secondary populations could be retained without any need for a direct, mutually beneficial interaction with the primary population. Second, it was possible that the community as a whole had the most competitive growth kinetic constants. In continuous-flow culture systems, the most important parameter is the saturation constant which measures the organisms' or the community's affinity for the limiting substrate (35). In monoculture the primary population had a saturation constant, K_s, of 100 mg orcinol liter^{-1}, whereas the complete mixed culture had a K_s of 71 mg orcinol liter^{-1}, indicating a significantly greater affinity for orcinol by the mixed culture than the primary population alone. This appeared to explain the selection of the mixed culture as compared with a pure culture of *P. stutzeri*. However, further experiments showed that a two-membered community of *P. stutzeri* and *Brevibacterium linens* exhibited an even greater orcinol affinity with a K_s of 56 mg orcinol liter^{-1}. This simpler community was not selected possibly because no growth conditions could be established leading to the competitive exclusion of the second secondary population, a species of *Curtobacterium*. The mechanism of interaction between the pseudomonad and the *B. linens* population, which seemed to be the significant relationship resulting in the increase or orcinol affinity, has not been elucidated.

THE VALUE AND POTENTIAL OF MIXED MICROBIAL POPULATION GROWTH

The examples cited previously have illustrated the value of specific interactions between different populations which have led to the formation of stable microbial communities. There are, however, considerable potential advantages to be gained from the growth of mixed populations in the same habitat, particularly when organisms have to respond to unusual or novel environmental conditions.

MIXED CULTURES WITH COMBINED METABOLIC OR COMETABOLIC CAPABILITIES

Mixed cultures may have a crucial role to fulfill in the degradation of complex natural products or xenobiotics. For example, several different microorganisms between them may have the complete capability to degrade a particular compound although none of the individual species have the full genetic information for the synthesis of all the necessary enzymes. Thus, under appropriate growth conditions a microbial community would be formed. One particular variation which may be important in nature, particularly for the degradation of unusual compounds, occurs if one member of the community is able to cometabolize a particular compound producing a metabolite which is then further metabolized by a second population.

Johanides and Hršak (24) have isolated a bacterial community growing on a mixture of at least twenty isomers and homologues of linear alkylbenzensulphonates which together comprised the growth-limiting substrate in a continuous enrichment. The resulting mixed culture predominantly contained *Pseudomonas* and *Alcaligenes* species in porportions which varied depending on the precise growth conditions. None of the isolated organisms were capable of growth on the mixed substrates in monoculture. It was suggested that the degradation of the surfactant depended on a combined metabolic attack by several organisms. It cannot, however, be eliminated at this stage that possibly some of the community interactions did not involve cometabolism of part of or the whole of the molecule (19) but specific nutritional interactions between the component populations.

Some years ago, Gunner and Zuckerman (14) described a synergistic relationship between a species of *Arthrobacter* and a *Streptomyces* species to account for the degradation of the insecticide Diazinon (0,0-diethyl 0-2-isopropyl-4-methyl-6-pyrimidyl thiophosphate) which strongly indicated that some form of combined metabolic attack was involved. Treatment of soil with the insecticide enriched these two organisms although individually, neither organism was able to utilize the compound as the sole carbon and energy source. The *Arthrobacter* species alone was able to metabolize the ethyl ester moiety but ^{14}C-labelled studies revealed that neither organism alone was able to metabolize the pyrimidine ring. As a community, however, rapid degradation occurred producing two unidentified metabolites which were not further degraded by these two organisms. Neither the mechanism of ring cleavage nor the precise enzymatic relationship between the two organisms was elucidated but these results have indicated the potential importance of such interactions in the degradation of complex compounds.

Another example of possible cooperative metabolism by two microorganisms comes from the leaching of iron and copper from sulphur-containing ores such as pyrite and chalcopyrite (1, 27). It has been shown that *Leptospirillum ferrooxidans* can generate energy from oxidation of ferrous iron but not from reduced sulphur compounds. Conversely, *Thiobacillus organoparus* cannot utilize ferrous iron but can obtain energy from the oxidation of reduced sulphur compounds. As pure cultures neither organism utilized pyrite or chalcopyrite while a mixed culture was able to rapidly oxidize the appropriate components of the ores. The nature of the interaction has not been explained.

Cometabolism occurs widely (18) and some recent evidence suggests that interactions based on cometabolism may be important in natural populations. Munneke et al. (29, 30) have adapted mixed populations to grow on the toxic insecticide parathion (0,0-diethyl 0-*p*-nitrophenyl phosphorothioate) using chemostat systems and isolated apparently stable multispecies communities. Most recently, Daughton and Hsieh (12) have characterized the metabolic core of one such community which had been selected after two years continuous growth on parathion. An initial examination suggested that the community apparently contained three organisms, none of which could hydrolyze parathion. A more reigorous examination of some of the slow-growing isolates revealed the presence of a fourth organism, *Pseudomonas stutzeri*, which had an extremely active parathion-hydrolyzing capacity. It was, however, unable to grow on either of the two hydrolysis products, namely diethyl thiophosphate and *p*-nitrophenol. One of the original isolates, *P. aeruginosa*, could grow on *p*-nitrophenol. Thus in the complete community, *P. aeruginosa* depended on the hydrolytic activity of the primary *P. stutzeri* which in turn depended on the provision of utilizable metabolites from the *P. aeruginosa* population. No role was ascribed to the two other non-parathion utilizing populations, a coryneform and an unidentified pseudomonad. None of the organisms were able to make use of the second breakdown product, diethyl thiophosphate.

Recently, using continuous-flow culture systems, we have isolated an unidentified mixed culture growing on a mixture of glycerol and dichloroacetic acid (DCA) as the combined growth-limiting substrates (Lovatt and Slater, unpublished data). At a growth rate of 0.1 h^{-1}, approximately 80% of the DCA was dechlorinated but DCA alone could not support the growth of the mixed culture in the chemostat and none of the organisms was able to grow individually on DCA. It seems probable that DCA was cometabolized although we have not completely excluded the possibility that the growth of a DCA utilizer depended on a nutritional interaction with another population in the mixed culture.

ADAPTATION TO GROWTH ON NOVEL CARBON SOURCES AND THE EVOLUTION OF DEGRADATIVE CAPABILITIES IN MIXED CULTURES

An interesting problem at the present time concerns the capacity of microorganisms to adapt to utilize novel carbon source for growth. For naturally occurring materials, microorganisms have had several billion years to evolve the necessary catabolic pathways. However, in the contemporary environment, microbial populations have been exposed to an increasing diverse range of unusual or environmentally foreign compounds over an exceedingly short period of time on an evolutionary scale. This situation raises the intriguing questions of how rapidly and by what mechanisms do microorganisms evolve the necessary, new catabolic potentials and whether or not there are any limitations to microbial degradative capabilities.

There are basically two classes of adaptation phenomena which have to be considered. First, the comparatively simple adaptations through mutation and appropriate selection, of existing catabolic mechanisms. Second, the more complex problems involved in the evolution of complete pathways for the metabolism of a new growth substrate. The former may involve changes in enzyme specificities, control and regulation mechanisms or transport systems for the assimilation of the new substrate (11). Pure culture studies with the aliphatic amidases of *Pseudomonas aeruginosa* (11) and the pentitol dehydrogenases of *Klebsiella aerogenes* (17, 41), have illustrated the importance of alterations at these levels in the acquisition of a new catabolic capacity. However, little is known in detail about the frequency and nature of adaptations at the molecular level in naturally occurring populations.

Senior et al. (34) isolated a multispecies community growing on the herbicide Dalapon, 2,2'-dichloropropionic acid (22DCPA). Although the original community contained three organisms growing on 22DCPA, it was observed that after a period of time one of the original secondary organisms, *Pseudomonas putida* S3, unable to grow on 22DCPA, acquired the potential to grow on the compound. Growth on chlorinated aliphatic acids required the presence of the enzyme dehalogenase and the new 22DCPA-utilizing strain, *P. putida* P3, was shown to contain 22DCPA specific dehalogenase activity with a maximum activity of 7.8 μmol 22DCPA converted/mg $\text{protein}^{-1}/h^{-1}$. Subsequently it was found that the parent strain could grow slowly on 2-monochloropropionic acid and under certain conditions very low levels of 22DCPA specific dehalogenase activity, approximately 0.5 µmol 22DCPA converted/mg protein^{-1}/h^{-1}. This adaptation involved either a change in the regulatory mechanisms or perhaps in the transport of 22DCPA. The important point is that the complete community provided a suitable environment, a "permissive" environment, for the retention of the parent organism in a situation where alone it would have been unable to grow and evolve.

More recently, we have examined the degradation of the herbicide lontrel, 3,6-dichloropicolinic acid (36DCPA) (Lovatt, Gilchrist and Slater, unpublished observations). It proved impossible to isolate organisms from soil, using either continuous-flow culture techniques or conventional dilution plates, able to grow on 36DCPA. We have, therefore, attempted to adapt a microbial community growing on picolinic acid to utilize 36DCPA. The original picolinic acid community was composed of three primary utilizers, *Pseudomonas aeruginosa*, *Alcaligenes faecalis* and a second *Alcaligenes* species, and three secondary organisms, namely *Bacillus licheniformis*, a species of *Rhodococcus*, and a *Corynebacterium aquaticum*-like organism. The community could not be grown on 36DCPA as the only carbon source and the initial attempts to adapt the culture to a mixture of picolinic acid and 36DCPA (in the ratio 5:1) also resulted in culture washout. However, after several cycles of growth on picolinic acid alone followed by a period of growth on the mixed substrates, the community adapted to grow in the presence of 36DCPA. From this culture, none of the organisms was able to grow on 36DCPA in pure culture although it was found that 36DCPA stimulated oxygen uptake in the mixed culture. There was no increase in the culture biomass suggesting that none of the 36DCPA carbon was utilized for biomass production. The composition of the 36DCPA-tolerant community, in terms of the primary organisms, was markedly different from that of the original community. Initially, at a growth rate of 0.05 h^{-1}, the community was dominated by *A. faecalis* at about 90% of the community, with *P. aeruginosa* the next most abundant primary picolinic acid-utilizer. In the adapted community considerable fluctuations in the primary populations were observed together with the second *Alcaligenes* species achieving an equivalent abundance with *A. faecalis*. We are examining the precise nature of this adaptation with one possibility being the selection of an organism with the potential to cometabolize 36DCPA.

THE APPLICATION OF CONTINUOUS-FLOW CULTURE TECHNIQUES TO THE STUDY OF MIXED CULTURES

Continuous-flow culture techniques provide the most suitable experimental systems for isolation and analysis of interacting mixed cultures. An important feature of continuous cultures is that the method ensures that interacting associations are selected provided that as a group the association was more competitive than individual organisms growing under the same conditions. Furthermore, one of the properties of open growth systems is that other organisms not involved in the required mixed culture are competitively selected against and rapidly eliminated from the growth vessel (34, 36). In addition, continuous-flow culture enrichments have an important advantage in great flexibility of the selection conditions which can be chosen. For example, by controlling the flow rate of fresh medium into the growth vessel, different growth rates may be predetermined. It is now well established from pure culture studies that this parameter is particularly significant in determining the outcome of continuous enrichments (15,21,22,25). It is also feasible to alter other environmental conditions such as the nature of the growth-limiting substrate and its concentration and physical parameters, although so far there have not been any systematic studies on the influence of these factors on the types of mixed culture selected for. Finally, open growth systems can be carefully controlled with the possibility that stable, steady-state conditions may be established (7).

The possibility of applying continuous-flow culture systems to problems in microbial ecology have been recognized for some time (e.g. 15,21) and the development of these techniques should provide valuable tools in attempting to understand the growth and behavior of microorganisms in nature. It can be argued that these culture systems can closely reflect the conditions occurring in nature and future developments, for example, developing systems able to contain particulate materials (8), should increase their relevance to such studies.

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LITERATURE CITED

1. Balashova, V. V., I. Y. Vedenina, G. E. Markosyan, and G. A. Zavarzin. 1973. The auxotrophic growth of *Leptospirillum ferrooxidans*. Mikrobiologiya 43:581-585.

2. Bates, J. L., and P. V. Liu. 1963. Complementation of lecithinase activities by closely related pseudomonads: its taxonomic implication. J. Bacteriol. 86:585-592.

3. Bergter, F., and D. Noack. 1966. Ein mikrobiologisches beispiel für oszillationen in einer zwel-arten-gesellschaft. Studia Biophys. 1:257-264.

4. Brock, T. D. 1966. Principles of microbial ecology. Prentice-Hall, Inc., Englewood Cliffs, N.J.

5. Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabill. 1977. Growth of *Desulfovibrio* in lactate or ethanol media low in sulphate in association with hydrogen utilizing methanogenic bacteria. Appl. Environ. Microbiol. 33:1162-1169.

6. Bryant, M. P., E. A. Wolin. M. J. Wolin, and R. S. Wolfe. 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch. Mikrobiol. 59:20-31.

7. Bull, A. T. 1975. Microbial growth, p. 415-442. *In* A. T. Bull, J. R. Lagnado, J. O. Thomas and K. F. Tipton (eds.), Companion to biochemistry.

8. Bull, A. T., and R. E. Ashby. 1977. A column fermenter for the continuous cultivation of microorganisms attached to surfaces. Lab. Prac. 26:327-329.

9. Cappenberg, Th. E. 1975. A study of mixed continuous cultures of sulphate-reducing and methane-producing bacteria. Microbial Ecol. 2:60-72.

10. Chao, C. C., and P. J. Reilly. 1972. Symbiotic growth of *Acetobacter suboxydans* and *Saccharomyces carlbergansis* in a chemostat. Biotech. Bioeng. 14:75-92.

11. Clarke, P. H. 1974. The volution of enzymes for the utilization of novel substrates, p. 183-217. *In* M. J. Carlile and J. J. Skehel (eds.), Evolution in the microbial world. 24th Symposium of the Society for General Microbiology.

12. Daughton, C. G., and D. P. H. Hsieh. 1977. Parathion utilization by bacterial symbionts in a chemostat. Appl. Environ. Microbiol. 34:175-184.

13. Gray, B. H., C. F. Fowler, N. A. Nugent, N. Rigopoulos, and R. C. Fuller. 1973. Reevaluation of *Chloropseudomonas ethylica*. Int. J. Syst. Bacteriol. 23:256-264.

14. Gunner, H. B., and B. M. Zuckerman. 1968. Degradation of 'Diazinon' by synergistic microbial action. Nature (London) 217:1183-1184.

15. Harder, W., and H. Veldkamp. 1971. Competition of marine psychophilic bacteria at low temperatures. Antonie van Leeuwenhoek 37:51-63.

16. Harrison, D. E. F., and S. Wren. 1976. Mixed microbial cultures as a basis for future fermentation processes. Process Biochem. 11:30-32.

17. Hartley, B. S. 1974. Enzyme families, p. 151-182. *In* M. J. Carlile and J. J. Skehel (eds.), Evolution in the microbial world. 24th Symposium of the Society for General Microbiology.

18. Horvath, R. S. 1972. Microbial co-metabolism and the degradation of organic compounds in nature. Bacteriol. Rev. 36:146-155.

19. Horvath, R. S., and B. W. Koft. 1972. Degradation of alkyl benzene sulfonate by *Pseudomonas* species. Appl. Microbiol. 23:407-414.

20. Ianotti, E. L., D. Kafkewitch, M. J. Wolin, and M. P. Bryant. 1973. Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: changes caused by interspecies transfer of hydrogen. J. Bacteriol. 114:1231-1239.

21. Jannasch, H. W. 1967. Enrichment of aquatic bacteria in continuous culture. Arch. Mikrobiol. 59:165-173.

22. Jannasch, H. W. 1968. Competitive elimination of *Enterobacteriaceae* from seawater. Appl. Microbiol. 16:1616-1618.

23. Jensen, H. L. 1957. Decomposition of chloro-substituted aliphatic acids by soil bacteria. Can. J. Microbiol. 3:151-164.

24. Johanides, V., and D. Hršak. 1976. Changes in mixed bacterial cultures during linear alkylbenzensulphonate (LAS) biodegradation, p. 426. *In* H. Dellweg (ed.), 5th International Symposium on Fermentation.

25. Kuenen, J. G., J. Boonstra, H. G. J. Schroder, and H. Veldkamp. 1977. Competition for inorganic substrates among chemoorganotrophic and chemolithotrophic bacteria. Microbial Ecol. 3:119-130.

26. Magee, R. D., J. F. Drake, A. G. Fredrickson, and H. M. Tsuchiya. 1972. Studies in intermicrobial symbiosis: *S. cerevisiae* and *L. casei*. Can. J. Microbiol. 18:1733-1742.

27. Markosyan, G. E. 1973. A new mixotrophic sulfur bacterium developing in acid media, *Thiobacillus organoparus* sp. N. Doklady Akademii Nauk SSSR 211:1205-1208.

28. Meers, J. L. 1973. Growth of bacteria in mixed culture. CRC Crit. Rev. Microbiol. 2:139-184.

29. Munneke, D. M., and D. P. H. Hsieh. 1974. Microbial decontamination of parathion and p-nitrophenol in aqueous media. Appl. Microbiol. 28:212-217.

30. Munneke, D. M., and D. P. H. Hsieh. 1975. Microbial metabolism of a parathion-xylene pesticide. Appl. Microbiol. 30:575-580.

31. Nurmikko, V. 1954. Symbiosis experiments concerning the production and biosynthesis of certain amino acids and vitamins in associations of lactic acid bacteria. Ann. Acad. Sci. Fenniciae 54:7-58.

32. Osman, A., A. T. Bull, and J. H. Slater. 1976. Growth of mixed microbial populations on orcinol in continuous culture, p. 124. *In* H. Dellweg (ed.), 5th International Symposium on Fermentation.

33. Schopfer, W. H. 1943. *In* Plants and vitamins. Chronica Botanica, Waltham, Mass.

34. Senior, E., A. T. Bull, and J. H. Slater. 1976. Enzyme evolution in a microbial community growing on the herbicide Dalapon. Nature (London) 263:476-479.

35. Slater, J. H., and A. T. Bull. 1978. Biochemical basis of microbial interactions. Ann. Appl. Biol. (in press).

36. Slater, J. H., and A. T. Bull. 1978. Interactions between microbial populations, p. 181-206. *In* A. T. Bull and P. M. Meadow (eds.), Companion to Microbiology.

37. Stirling, L. A., R. J. Watkinson, and I. J. Higgins. 1976. The microbial utilization of cyclohexane. Soc. Gen. Microbiol. 4:28.

38. Wilkinson, T. G., H. H. Topiwala, and C. Hamer. 1974. Interactions in a mixed bacterial population growing on methane in continuous culture. Biotech. Bioeng. 16:41-47.

39. Wilson, T. E., and P. W. Goaz. 1959. The oral yeast-*Lactobacillus* relationship. J. Dent. Res. 38:1044-1051.

40. Wolfe, R. S., and N. Pfennig. 1977. Reduction of sulphur by *Spirillum* 5175 and syntrophism with *Chlorobium*. Appl. Environ. Microbiol. 33:427-433.

41. Wu, T. T., E. C. C. Lin, and S. Tanaka. 1968. Mutants of *Aerobacter aerogenes* capable of utilizing xylitol as a novel carbon source. J. Bacteriol. 96:447-456.

42. Yeoh, H. T., H. R. Bungay, and N. R. Kreig. 1968. A microbial interaction involving a combined mutualism and inhibition. Can. J. Microbiol. 14:491-492.

PREDICTING THE FATE OF ORGANICS: CONCEPTUAL MODEL AND EXPERIMENTAL APPROACHES

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ABSTRACT

A procedure for systematically developing a predictive model for synthetic organics is demonstrated and discussed. Specifically a conceptual model illustrating the pathways of an organic in an aquatic ecosystem is presented. This conceptual model is further translated into a time-varying mathematical framework. This potential predictive model requires extensive field and laboratory experimentation. Two experimental approaches, a Dual Culture Diffusion Apparatus and a Two-Stage Continuous Flow System, are suggested for providing certain descriptive and kinetic data necessary to the predictive model.

INTRODUCTION

There exists a massive number of industrially generated synthetic organics, possessing widely different chemical natures, which are released into our ecosystem. Eight hundred million pounds of pesticides are applied annually in the United States and their use has risen rapidly throughout the world. Presently, the external costs of using these pesticides may be estimated as high as two to three billion dollars annually and identifiable human pesticide poisonings are nearly 60,000 per year (18). Over 700 synthetic organics have so far been identified in drinking waters and many of these chemicals have been found to be carcinogenic in animals or to exhibit toxic properties (1). Many of these organics (PCBs and DDT, for example) have been detected in many remote areas of the world (8, 16, 17, 22, 24), indicating that these organics may have universally contaminated our biosphere.

Thus far, the mechanisms responsible for the transport of synthetic organics are not accurately described and quantified. Even the analytical measurement and biological consequences of these organics are not fully elucidated. The present situation requires effective methods for evaluating the potential hazards of the many organics now being produced. The development and application of a deterministic model, simulating the natural phenomena affecting the fate of organics within a mathematical framework, would facilitate the necessary evaluations.

There currently exists a number of conceptual and developing mathematical models intended to suggest the fate of organics (7, 9, 16, 24). The conceptual models have succeeded in identifying and assimilating many potential pathways of synthetic organics. For the most part, these general pathways are the same, regardless of whether a given organic is of natural or synthetic origin.

In the process of translating these conceptual models to descriptive, deterministic models it becomes evident that although the pathways may be similar the experimental quantification of controlling processes and the subsequent calibration of the model are the two remaining tasks in developing predictive capabilities. The kinetics which govern these processes remain, for the most part, unknown quantities requiring comprehensive investigation.

The scope of this paper is to demonstrate a methodology for constructing a model describing the fate of organics. This model emphasizes the need for experimental work and the necessity for kinetic data. The paper offers two experimental apparatus which may be utilized for quantifying some of the processes.

CONCEPTUAL MODEL

A conceptual model recognizes known concepts of an ecosystem and proceeds to translate them into an interacting functional diagram (9). Conceptual models are the first step in organizing scientific knowledge and hypothesis for the purpose of predicting the fate and effects of an agent released into the environment. These models may provide answers to questions concerning the variables requiring measurement, the accuracy required of the measurement, and the frequency and location of sampling (9). Conceptual models also serve as a guideline for the development of mathematical models.

The construction of a conceptual model describing the fate of organics requires the identification of pertinent phenomena. In Table 1 we see a synopsis of the relevant processes, the parameters influencing these processes, and the compartments in which they are active. The specific nature of an organic will determine which processes and compartments will be utilized in a particular model.

For a functional diagram to be representative of a given ecosystem's dynamics and compatible with existing mathematical frameworks, it is often desirable to further divide compartments into smaller box models (3, 6, 14, 23). A conceptual representation of a two-box model describing the fate of an organic within a lake ecosystem is illustrated in schematic form in Figure 1. This schematic model represents a summer stratified lake, which includes all the processes presented in Table 1. The upper box represents the epilimnion and the lower box the hypolimnion. Within each box a given organic may be either bound in a solid phase or in free solution. Both boxes also contain their own set of relevant sources and sinks for each organic form. Mass transport between boxes takes place by diffusion and/or sedimentation across the thermocline.

TABLE 1. SYNOPSIS OF PROCESSES AFFECTING THE FATE OF ORGANICS

Process	Controlling parameters	Compartment
Photodecomposition	Chemical Structure, Ultraviolet Intensity	Soil-Atmospheric-Aquatic
Evaporation	Activity Coefficients, Partial Vapor Pressure, Diffusion Rates, Desorption Rates, Temperature	Soil-Atmospheric-Aquatic
Leaching	Hydrodynamic Diffusion and Dispersion, Adsorption-Desorption, Degradation Rates, Evaporation	Soil
Adsorption-Desorption	Heat of Adsorption, Functional Groups, Surface Area, Water Solubility of Organic, Organic Content of Soil pH and Temperature	Soil-Atmospheric-Aquatic
Dispersion	Advection, Diffusion, Aerosol Properties, Chemical Reactions, Meteorological Factors Topographical Features	Atmospheric
Bacterial Degradation	Chemical Nature of Organics, Diffusion, Adsorption, Temperature, Microbial Dynamics.	Soil-Aquatic
Chemical Decay	Chemical Structure, Catalyzers, pH, Temperature	Soil-Atmospheric-Aquatic
Cultural Inputs	Agricultural, Industrial, and Political Practices	Soil-Atmospheric-Aquatic

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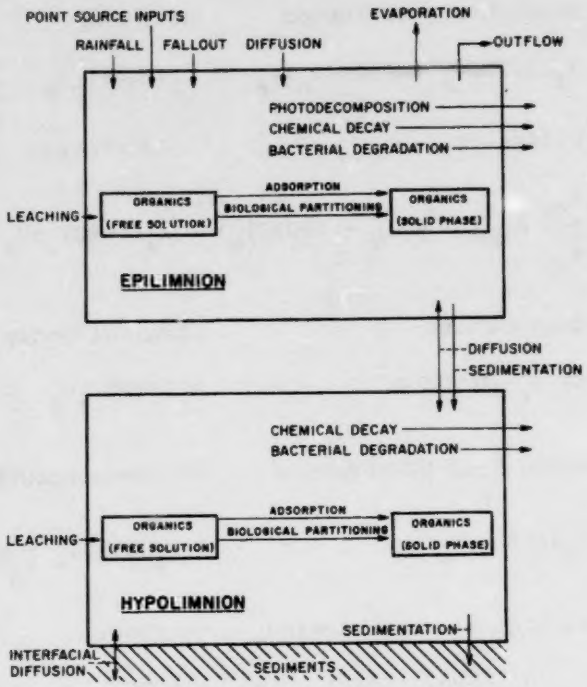

Figure 1. Schematic outline of two-box organics model for lakes.

DETERMINISTIC MODEL

After gaining a conceptual grasp of the organic pathways in an ecosystem, it is necessary to further translate this scientific knowledge to a mathematical representation. The time-varying fate of an organic compound is mathematically described by formulating a mass balance, which includes the processes listed in Table 1.

This mass balance approach has been used to translate the conceptual two-box model in Figure 1 into a mathematical framework. Each process within a box is represented as a mathematical term. Since there are two organic forms (the solid phase [PORG] and the solution phase [SORG]) and each may reside in two boxes (epilimnion and hypolimnion), a complete mass balance results in four interdependent differential equations (22). (Nomenclature at end of paper.)

[SORG] in the Epilimnion (eq. 1)

$$V_e \frac{d[\mathrm{SORG}]_e}{dt} = \overset{\text{Sum of Point Inputs}}{\Sigma\, Q_n [\mathrm{SORG}]_n} \quad + \overset{\text{Rainfall or Washout}}{I\, A_e [\mathrm{SORG}]}$$

Atmospheric Diffusion   Leaching

$$+ k_f([SORG]_s - [SORG]_e)V_e \quad + k_L[SORG]_jV_e$$

Diffusion   Adsorption

$$+ \frac{k_{th}}{\bar{z}_{th}} A_{th}([SORG]_h - [SORG]_e) \quad - k_a[SORG]_e\theta V_e$$

Evaporation   Chemical Decay

$$- EP_iM_e \cdot 10^6/18GP_w \quad - \quad k_c[SORG]_eV_e$$

Biological Degradation   Photodecomposition

$$- k_d[SORG]_eV_e \quad - \quad V_ek_p[SORG]_e \frac{1}{k_gH}[e^{-\alpha 1} - e^{-\alpha 0}]$$

Biological Partitioning   Outflow

$$- \Sigma\ U[Biomass]_e[SORG]_e \quad - \quad Q[SORG]_e$$

[PORG] in the Epilimnion   (eq. 2)

Sum of Point Inputs   Rainfall or Washout

$$V_e \frac{d[PORG]_e}{dt} = \Sigma\ Q_n\ [PORG]_n \quad + I\ A_e[PORG]_r$$

Fallout   Adsorption

$$+ Mf \cdot A_e \quad + k_a[SORG]_eV_e$$

Biological Partitioning   Diffusion

$$+ \Sigma\ U[Biomass]_e[SORG]_e \quad + \frac{k_{th}}{\bar{z}_{th}} A_{th}([PORG]_h - [PORG]_e)$$

Sedimentation   Biological Degradation

$$- g_eA_{th}\ [PORG]_e \quad - \Sigma\ k_d\ [PORG]_eV_e$$

Chemical Decay   Outflow

$$- \Sigma\ k_c\ [PORG]_eV_e \quad - Q[PORG]_e$$

[SORG] in the Hypolimnion (eq. 3)

Diffusion Leaching

$$V_h \frac{d[\mathrm{SORG}]_h}{dt} = \frac{k_{th}}{\bar{z}_{th}} A_{th}([\mathrm{SORG}]_e - [\mathrm{SORG}]_h) + k_L[\mathrm{SORG}]_j V_h$$

Adsorption Biological Degradation

$$- k_a[\mathrm{SORG}]_h \theta V_h \qquad - k_d[\mathrm{SORG}]_h V_h$$

Biological Partitioning Chemical Decay

$$- \Sigma\, U[\mathrm{Biomass}]_h[\mathrm{SORG}]_h \qquad - k_c[\mathrm{SORG}]_h V_h$$

Interfacial Diffusion

$$+ k_i A_{sd}([\mathrm{SORG}]_j - [\mathrm{SORG}]_h)$$

[PORG] in the Hypolimnion (eq. 4)

Diffusion Sedimentation

$$V_h \frac{d[\mathrm{PORG}]_h}{dt} = \frac{k_{th}}{\bar{z}_{th}} A_{th}([\mathrm{PORG}]_e - [\mathrm{PORG}]_h) + g_e A_{th}[\mathrm{PORG}]_e$$

Adsorption Biological Degradation

$$+ k_a[\mathrm{SORG}]_h \theta V_h \qquad + \Sigma\, U[\mathrm{Biomass}]_h[\mathrm{SORG}]_h$$

Biological Degradation Chemical Decay

$$- \Sigma\, k_d[\mathrm{PORG}]_h V_h \qquad - \Sigma\, k_c[\mathrm{PORG}]_h V_h$$

Loss to Sediments

$$- g_h A_{sd}\, [\mathrm{PORG}]_h$$

Similar equations to the ones above could be derived for a non-stratified winter model, which would be represented by a one-box model. The summer and winter models would be joined by boundary conditions existing at fall overturn and spring stratification. At the fall overturn the [SORG] would be

$$\frac{[\mathrm{SORG}]_e\, V_e + [\mathrm{SORG}]_h\, V_h}{V} \qquad \text{(eq. 5)}$$

and the [PORG] would be

$$\frac{[PORG]_e V_e + [PORG]_h V_h}{V} \qquad \text{(eq. 6)}$$

The concentrations of the epilimnetic and hypolimnetic organics, $[SORG]_e$, $[SORG]_h$, $[PORG]_e$, $[PORG]_h$, are those calculated at the end of the stratified period (or Two-Box Model). These concentrations at the beginning of stratification are set equal to the concentration of organics ([PORG] or [SORG]) at the conclusion of the winter one-box model.

Any given mathematical representation offered in the differential equations is for the present a hypothetical representation and is not meant to be a definitive statement. However, some of these terms have been shown in the literature to be a good representation of the process. For instance, Snodgrass and O'Melia (23) had success with their representation of diffusion and sedimentation (which took into account lake depth) when developing a model to predict the fate of phosphorus in Lake Ontario. The term used for evaporation was developed by Mackay et al. (15) and provides a method for calculating the potential evaporation for compounds whose vapor pressure and water solubility are known. The adsorption term used in the model was formulated by Hamaker and Thompson (11). Hamaker (10) also suggested the leaching term which depends strictly on empirical data. Photodecomposition rates could vary as a function of light (or UV) intensity; therefore, a light extinction factor utilized in phytoplankton models was employed (3).

Inspection of the deterministic model presented in equations 1 through 4 reveals that it is necessary to quantify many parameters and that knowledge of the kinetics of these processes is of utmost importance. Kinetic parameters needing experimental evaluation include determination of the rate coefficients describing bacterial degradation, adsorption, biological partitioning, chemical decay, photodecomposition and leaching.

Experimentation dealing with certain processes may reveal that additional parameters may have to be treated as state variables and modeled separately. One such process may be the microbial degradation of organics. The dynamics of microbial populations may greatly affect the extent and rate of organic decomposition. The specific assemblage of microorganisms will certainly determine the by-products of decomposition. Environmental perturbations that would not directly affect a specific organic pool could certainly effect the microbial dynamics which in turn would influence the fate of the organic.

The inclusion of bacteria as a state variable in a model describing the fate of organics requires extensive experimental efforts to quantify processes such as: the uptake rates of soluble organics; bacterial specific growth rates as a function of nutrient utilization; stoichiometric bacterial biomass production from soluble organics; the rate of bacteria-mediated by-product formation; and the ability of bacteria to utilize organics adsorbed on particulate matter. Several investigators have recently developed models which are intended to model microbial growth and decomposition activity within an aquatic ecosystem (2, 4, 5, 13). In order to utilize and to improve these microbial growth models extensive field and laboratory investigation must be initiated in order to supply specific data for the various groups of synthetic organics.

EXPERIMENTAL APPROACHES

Two experimental approaches which lend themselves to process research and kinetic evaluation of organics in natural water are proposed and discussed in this paper. The first approach employs a Dual Culture Diffusion Apparatus (DCDA) (Fig. 2). The DCDA allows two batch cultures to share the same media while keeping non-soluble substances separated by a polycarbonate membrane.

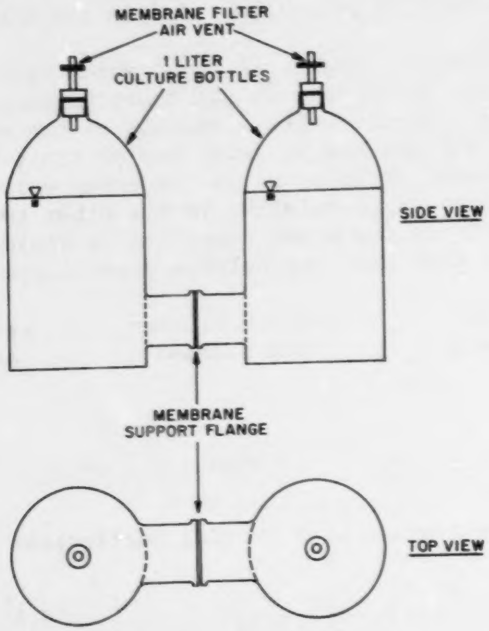

Figure 2. Diagram of Dual Culture Diffusion Apparatus (DCDA).

Thus far the DCDA has been utilized to measure the rate of inorganic phosphorus regeneration due to bacteria-mediated and endogenous decay of phytoplankton. The general approach has been to maintain one side of the DCDA in the dark (the culture vessel) and the adjoining side in an appropriate lit environment (the assay vessel). The culture vessel supported a previously grown algal culture, *Scenedesmus* sp., with or without a bacterial community present. The assay vessel was then inoculated with a phosphorus-starved *Scenedesmus* culture and maintained in the light. The intent of the experimental design was to measure the rate of phosphorus mineralization that might occur in the dark culture vessel via bacterial decomposition and algal respiration. To accomplish this purpose phosphorus fractions were periodically sampled in the assay vessel. Any increase in total phosphorus was assumed to have originated from mineralization in the culture vessel which had subsequently diffused through the membrane and had been accumulated by the P-starved *Scenedesmus*. By calculating a mass balance on phosphorus in both vessels of the DCDA regeneration rates of phosphorus could be obtained. This experimental approach has two primary advantages over previous nutrient regeneration studies. In batch cultures it has been found that nutrient uptake is a competitive parallel reaction with nutrient regeneration. Therefore, sampling a classical batch culture for regenerated soluble nutrients allowed only the net regeneration rates to be obtained (21). In comparison, the DCDA maintains a competitive rate with nutrient uptake in the culture vessel, namely, a faster rate of diffusion of soluble nutrients. When these diffused nutrients are immobilized in the assay vessel, rates which more closely represent gross regeneration rates are obtainable. This technique also demonstrates directly that by-products of degradation (in the culture vessel) are subsequently capable of being reassembled into algal biomass (in the assay vessel).

The DCDA functionally has three rates which ultimately determine the change in particulate phosphorus in the assay culture: a) the rate of phosphorus mineralization in the culture vessel; b) the rate of diffusion across the membrane which divides the vessels; and c) the rate of uptake of phosphorus by the P-starved *Scenedesmus* in the assay vessel. Phosphorus uptake was assumed to be very rapid relative to the other two rates and the presence of soluble phosphorus in the assay vessel to be minimal at all times (18). A mass balance on the dark side (or culture vessel) appears in equations 7 and 8.

$$\begin{array}{c} \text{Accumulation of} \\ \text{soluble phosphorus} \\ (C_1) \end{array} = \begin{array}{c} \text{rate of release} \\ \text{from biomass} \end{array} - \begin{array}{c} \text{rate of diffusion} \\ \text{across membrane} \end{array} \qquad \text{(eq. 7)}$$

$$V \frac{dC_1}{dt} = W(t) - \frac{DAC_1}{\sigma} \qquad \text{(eq. 8)}$$

For first order regeneration with initial particulate phosphorus = P_o.

$$C_1(t)\ V = P_o\ V \left(1-e^{-k_r t}\right) \qquad \text{(eq. 9)}$$

therefore the rate of release from the biomass would be

$$W(t) = k_r\ P_o\ V\ e^{-k_r t} \qquad \text{(eq. 10)}$$

Substituting eq. 10 into eq. 8, the material balance becomes

$$\frac{DC_1}{dt} + \alpha C_1 = k_r\ P_o\ e^{-k_r t} \qquad \text{(eq. 11)}$$

where $\alpha = \frac{DA}{\sigma V}$

The solution to eq. 11 for $C_1 = 0$, when $t = 0$ is

$$C_1(t) = \frac{k_r P_o}{(\alpha - k_r)} \left[e^{-k_r t} - e^{-\alpha t} \right] \qquad \text{(eq. 12)}$$

Since it is the lit side (or assay vessel) which is sampled during an experiment it is necessary to formulate a mass balance on the lit vessel for the accumulation of algal particulate phosphorus (C_2), presented in equations 13 and 14:

Rate of C_2 accumulation = rate of C_1 diffusion across membrane (eq. 13)

$$V \frac{dC_2}{dt} = \alpha C_1 \qquad \text{(eq. 14)}$$

Solution to eq. 14 for $C_2 = 0$, when $t = 0$ is

$$C_2(t) = \frac{P_o}{(\alpha - k_r)} \left[1 - e^{-k_r t} \right] - \frac{k_r P_o}{(\alpha - k_r)} [1 - e^{-\alpha t}] \qquad \text{(eq. 15)}$$

If the value of $\alpha >> k_r$ then eq. 15 reduces to

$$C_2(t) = P_o \left[1 - e^{-k_r t} \right] \qquad \text{(eq. 16)}$$

Equation 16 simply states that if the diffusion rate is significantly greater than the release rate then the accumulation of particulate phosphorus (C_2) in the lit side would not be diffusion limited. In Figure 3 a graphical comparison of eq. 15 and 16, using laboratory data, is seen. In this case the lag in phosphorus accumulation in the assay vessel is the result of some diffusion limitation and not due to a lag in phosphorus regeneration in the culture vessel.

Experimentation has shown that the rate of diffusion, α, from the culture vessel (volume = 900 ml) across the membrane to the assay vessel of low levels of phosphate (10-150 µgP/liter) is as high as 0.4 day^{-1}. By utilizing the mass balance scheme presented above and assuming that $\alpha >> k$ (utilizing eq. 16), we obtained first-order phosphorus regeneration rates that varied from 0.001-0.05 day^{-1}. The rates appear to be dependent on the physiological state of the algae and the size fractionation of the decomposer community.

We believe that the DCDA experimental approach presented above offers a method for evaluating certain processes that determine the fate of organics in aquatic ecosystems. Adsorption or desorption rates could be measured without disturbing the vessel housing the process of interest. Suppose a certain particle has been saturated with a particular organic and is placed in the culture vessel. Then an adsorbent with no sites previously occupied is placed in the assay vessel, which is sampled periodically. By utilizing the previous mass balance approach the rate of adsorption in the assay vessel can be used to calculate the rate of deadsorption in the culture vessel.

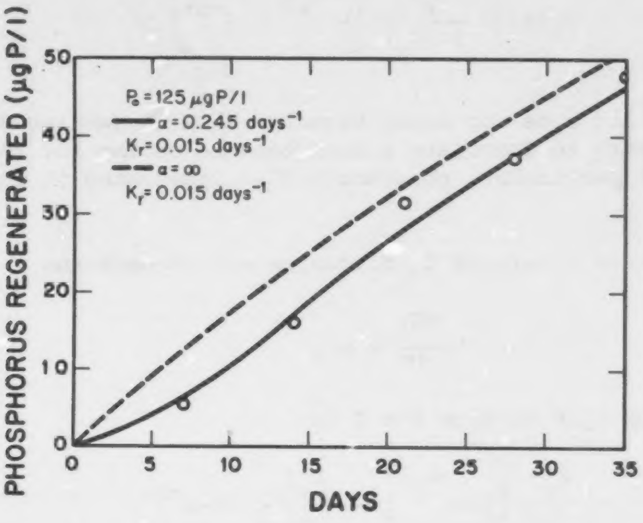

Figure 3. Phosphorus regeneration from aerobic decomposition of *Scenedesmus* in a DCDA. Dashed and solid lines represent the predicted phosphorus accumulation in the assay vessel for a first-order regenerarate of 0.015 day^{-1} and diffusion rates of ∞ and 0.245 day^{-1}, respectively.

Adsorption rates could also be investigated in less time and utilizing fewer vessels by placing the adsorbent in the culture vessel and spiking the vessels with a known quantity of organic and monitoring the decrease of organics in the assay vessel compared to a control. This approach would be feasible only if the rate of adsorption was not significantly higher than the rate of diffusion. Rates of adsorption for 2,4-D have been found to vary from 1.728×10^{-2} day^{-1} to 3.8×10^{-1} day^{-1} depending on the adsorbent clay (11).

Since specific by-product formation is highly dependent upon the particular microbial population and environmental conditions, the DCDA would offer a method of study that would not require harvesting or disturbing the decomposing culture. If the decomposing culture, including the original spike of organic, were housed in the culture vessel then the assay vessel could be periodically sampled and replaced with fresh media. The withdrawn media could be analyzed for the by-products while the decomposing culture is never disturbed.

Other possible organic process-related applications of the DCDA include: microbial mediated recycle or transformation of organics bound to particles; biological partitioning and microbial degradation or release of these partitioned organics; and possibly certain species interactions. Hopefully additional applications will be recognized where separation of process phases are desirable or where it would be advantageous not to disturb or harvest a culture in order to evaluate its dynamics.

A second experimental apparatus which lends itself to the study of organic kinetics is the continuous-flow apparatus. A continuous-flow culture offers several advantages over a batch culture. Since steady-state is reached, a mass-balance describing the process is simplified from a differential equation to a linear algebraic equation where kinetic data can be more easily extracted. To illustrate this point a hypothetical mass balance describing the decomposition and endogenous respiration of algae results in

$$V \frac{dA}{dt} = Q (A_o - A) - V N A - \frac{\mu_B B}{Y} V \qquad \text{(eq. 17)}$$

By defining Q/V = D (dilution rate) and considering a steady-state situation (i.e., dA/dt = 0) the mass balance becomes

$$0 = D (S_o - S) - N S - \frac{\mu_B B}{Y} \qquad \text{(eq. 18)}$$

Clearly the above equation is easily analyzed and offers a number of opportunities for linearization. This system allows extraction of kinetic data which is directly applicable to time-varying models such as the deterministic model presented previously.

Our experimental work has expanded the classical flow-through design to include two stages. The first stage is fed algal growth media at a constant rate and overflow. Steady-state is evaluated with in vivo Chlorophyll "a", electronic particle counts and particle volume concentration determinations. The spent media from the first-stage flow-through growth culture overflows into a second-stage flow-through culture where growth is prohibited by a darkened environment and algal respiration and bacterial metabolic activity influences a change in total biomass and physiological state of the algae.

Two-stage continuous-flow cultures could be employed to simultaneously follow phytoplankton uptake of a given organic compound in the first stage and bacteria-mediated release and/or degradation of the biologically bound (partitioned) organic in the second stage.

The continuous-flow system also allows the investigator to assay the effect an organic would have on the growth rate, physiological state and nutrient utilization of a range of microorganisms. This is accomplished because the feed characteristics and dilution rate force a culture with a defined mean physiological state.

Furthermore, the continuous-flow system offers a system which would facilitate the modeling of bacteria as a state variable. The growth rates and population dynamics could be obtained as a function of the feed composition. Media characteristics, which might include presence of by-products and persistence of different forms of organics as a function of residence time and microbial assemblage, could be measured. The chemostate has long been used for evaluation of bacterial growth kinetics and has the potential for assisting the development of a sub-model describing the dynamics of a decomposer community within an aquatic system.

SUMMARY

Because of the ever-increasing production of new synthetic organic chemicals, the prediction of the fate of these materials in our ecosystems has become a health and economic necessity. In this paper we have shown that a mathematical ecological model offers an efficient method of tracing the fate of synthetic organics discharged into aquatic environments. The usefulness of the model proposed herein is twofold. First, it may be used to correlate field and laboratory experimental data. In this way the model can be used as a research tool to establish a feedback loop between process experimentation and overall model development. Second, the verified model provides a basis for making informed decisions regarding agricultural, industrial, and municipal management of synthetic organic chemicals.

Further refinement and development of the proposed model will not only include evaluation of rate coefficients, but expansion of the scope of the model to include specific biological functional groups (such as bacteria, phytoplankton, and zooplankton) as state variables. In this way we can better predict the fate of an organic in addition to evaluating any perturbation of the aquatic community due to the compound in question. The ultimate goal of such a model is to be able to make management decisions regarding a given synthetic chemical before it is released into the environment.

Another goal of this paper was to point out the pressing need for kinetic data for the development of models such as the one proposed herein. The DCDA and the continuous-flow system (single or multi-stage), whose operation has been demonstrated, are experimental tools which may provide a method for characterizing and quantifying some of the processes governing the fate of organics. These experimental techniques combined with many others, in the laboratory and in the field, will make the development of a predictive model for organics a feasible goal.

ACKNOWLEDGMENT

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NOMENCLATURE

A = surface area, L^2
E = water evaporated, M/T
e = epilimnion subscript
g = sedimentation coefficient, L/T
G = grams of water containing M_e, M

h = hypolimnion subscript
H = depth of euphotic zone, L
I = intensity of rain, L/T
I_a = light intensity at surface
I_s = optimum intensity at surface
j = soil origin subscript
k_a = adsorption rate constant, 1/T
k_c = first order chemical decay coefficient, 1/T
k_d = first order biological degradation coefficient, 1/T
k_f = overall diffusion coefficient, 1/T
k_g = extinction coefficient of light (or UV), M/L^3
k_i = mass transfer coefficient, L/T
k_L = (cm moved by organic)/(cm of water entering the soil)(time), 1/T
k_p = first order photodecomposition coefficient, 1/T
k_{th} = mean vertical eddy diffusion coefficient, L^2/T
L = length
M = mass
M_e = grams of organic compound, M
Mf = fallout flux, $M/L^2 \cdot T$
n = input from point sources subscript
N = endogenous respiration rate, 1/T
P = vapor pressure of [illegible] compound (mm Hg)
P_w = vapor pressure of [illegible] Hg)
PORG = organics in particulate or solid phase
Q = flow, L^3/T
r = rain input subscript
S = saturation value subscript
sd = sediment subscript
SORG = organics in free solution phase
T = time
th = thermocline subscript
U = second order accumulation rate, $L^3 \cdot L^3/M \cdot T$
V = volume, L^3
$\bar{z}_{th}$ = average depth of thermocline, L
[ ] = concentration, M/L^3
θ = the fraction of adsorptive area uncovered
$\alpha 0$ = I_a/I_s
$\alpha 1$ = $I_a/I_s \exp(-k_g \cdot h)$

LITERATURE CITED

1. Anonymous. 1978. EPA proposes far-reaching regulations for reducing synthetic organics in drinking water. Series on Water Resources: No. 5, Civil Eng. ASCE, p. 69-71.

2. Barsdate, R. J., R. T. Prentki, and T. Fenchel. 1974. Phosphorus cycle of model ecosystems: significance for decomposer flood chains and effect of bacterial grazers. Oikos 25:239-251.

3. Bierman, V. J., Jr. 1976. Mathematical model of the selective enhancement of blue-green algae by nutrient enrichment, p. 1-32. *In* R. P. Canale (ed.), Modeling biochemical processes in aquatic ecosystems. Ann Arbor Science Publishers, Ann Arbor, Michigan.

4. Clesceri, L. S., R. A. Park, and J. A. Bloomfield. 1977. General model of microbial growth and decomposition in aquatic ecosystems. Appl. Environ. Microbiol. 33:1047-1058.

5. Cordeiro, C. F. 1975. Mathematical modeling of lake systems with nutrient cycling. Ph.D. dissertation, University of Notre Dame, Notre Dame, Ind.

6. Crews, W. B., J. W. Brewer, and T. J. Petersen. 1975. Modeling of atmospheric behavior: a submodel of the dynamics of pesticides, p. 79-96. *In* R. Haque and V. H. Freed (eds.), Environmental dynamics of pesticides. Plenum Press, New York.

7. Davidson, J. M., and J. R. McDougal. 1973. Experimental and predicted movement of three herbicides in a water-saturated soil. J. Environ. Quality 2:428-433.

8. Durham, W. F., and J. F. Armstrong. 1961. Insecticide content of diet and body fat of Alaskan natives. Science 134:1880-1881.

9. Gillett, J. W., J. Hill IV, A. W. Jarvinen, and W. P. Schoor. 1974. A conceptual model for the movement of pesticides through the environment. Ecological Research Series, U.S. Environmental Protection Agency (EPA-660/3-74-024).

10. Hamaker, J. W. 1975. The interpretation of soil leaching experiments, p. 115-133. *In* R. Haque and V. H. Freed (eds.), Environmental dynamics of pesticides. Plenum Press, New York.

11. Hamaker, J. W., and J. M. Thompson. 1977. Adsorption, p. 49-144. *In* C. A. I. Goring and J. W. Hamaker (eds.), Organic chemicals in the soil environment, Vol. 1. Marcel Dekker, Inc., New York.

12. Haque, R., F. T. Lindstrom, V. H. Freed, and R. Secton. 1968. Kinetic study of the sorption of 2,4-D on some clays. Environ. Sci. Technol. 2:207-211.

13. Krogius, F. V., E. M. Krokhin, and V. V. Menshutkin. 1972. The modeling of the ecosystem of Lake Dalnee on an electronic computer, p. 149-164. *In* Z. Kajak and A. Hillbricht-Dkowska (eds.), Productivity problems of fresh waters.

14. MacCracken, M. C., T. V. Crawford, K. R. Peterson, and J. B. Knox. 1971. Development of a multi-box air pollution model and initial validation for the San Francisco Bay Area. URCL-73348, Lawrence Radiation Laboratory, Livermore, California.

15. Mackay, D., and W. W. Wolkoff. 1973. Rate of evaporation of low-solubility contaminants from water bodies to atmosphere. Environ. Sci. Technol. 7:611-614.

16. Nisbet, I. C. T., and A. F. Sarofim. 1972. Rates and routes of transport of PCBs in the environment. Environ. Health Perspect. 1:21.

17. Peterle, T. J. 1969. DDT in Antarctic show. Nature 224:620.

18. Pimentel, D. 1978. Benefits and costs of pesticide use in U.S. food production. Unpublished MS.

19. Rhee, G-Y. 1973. A continuous culture study of phosphate uptake, growth rate and polyphosphate in *Scenedesmus* sp. J. Phycol. 9:495-506.

20. Risebrough, R. W., R. J. Huggett, J. J. Griffin, and E. D. Goldberg. 1968. Pesticides: transatlantic movements in the northeast trades. Science 159:1233-1236.

21. Rodgers, P. W., and J. V. DePinto. 1976. Laboratory study of freshwater algae-bacteria interactions and accompanying nutrient dynamics. Master's thesis, Clarkson College of Technology, Potsdam, N.Y.

22. Sladen, W. J. L., C. M. Menzie, and W. L. Reichel. 1966. DDT residues in Adelie Penguins and a Crabeater Seal from Antarctica. Nature 210: 670-673.

23. Snodgrass, W. J., and C. R. O'Melia. 1975. Predictive model for phosphorus in lakes. Environ. Sci. Technol. 9:937-944.

24. Woodwell, G. M., P. P. Craig, and H. A. Johnson. 1971. DDT in the biosphere: where does it go? Science 174:1101-1107.

TRANSPORT AND FATE OF ANTHRACENE IN AQUATIC MICROCOSMS[†]

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAH), many of which are known carcinogens, are expected to be important components of wastewater from coal conversion facilities. No large-scale conversion facilities are currently in operation, and information about the environmental behavior of PAH is scarce. Microcosms which mimic the structure and function of pond ecosystems can provide some of this information. Two 80-liter pond microcosms were treated with 0.5 μg/liter [9-^{14}C] anthracene, a representative PAH, to measure: the rate of sorption by sediments, bioaccumulation by several aquatic organisms, and transformation of anthracene by abiotic processes and by aquatic organisms, including bacteria. Samples of water, sediment, and organisms were removed periodically for determination of ^{14}C activity. Additional samples were extracted with organic solvents for separation and quantitation of anthracene and transformation products by thin-layer chromatography and radioautography. Anthracene disappeared rapidly from pond water but accumulated in sediments. Over a 12-week period, 80% of the anthracene was transformed, mainly by photolysis and biological activity. An unidentified derivative of anthracene persisted in all microcosm components examined, while anthracene and another degradation product persisted in the upper layer of sediment.

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INTRODUCTION

A major aspect of our national energy program is development of technologies for converting coal to liquid and gaseous fuels. The aqueous effluents from coal conversion facilities are expected to include hundreds or thousands of organic compounds, including phenols, aromatic amines, monoaromatic hydrocarbons, thiophenes, and polycyclic aromatic hydrocarbons (PAH). PAH are of particular concern for several reasons: (a) many are carcinogenic; (b) they may not be removed efficiently by biological waste treatment; (c) some appear to be resistant to degradation; (d) their environmental fate is practically unknown (4).

We have selected anthracene, a 3-ring PAH, for intense study as a representative compound of this class. Compared with other PAH, anthracene is intermediate in size (M.W. = 188), solubility in water (73 μg/liter, [6]), and octanol:water partitioning coefficient (2×10^4, [5]). The characteristics of anthracene which determine its fate in aquatic environments have been summarized by Southworth (7). Anthracene is moderately volatile, with a predicted volatilization half-life of 300 h in a 1-m deep water column under quiescent conditions. It sorbs strongly to suspended particulate organic matter (K_D = concentration on particulates/concentration in water = 25,000), an order of magnitude less to clay particles (K_D = 1600), and only slightly to silt particles (K_D = 100) (7). Anthracene is rapidly degraded in sunlight. The photolytic half-life in a 2-cm column of distilled water is approximately 35 min under midday midsummer sunlight at 35°N. Because photolysis is a function of light intensity, absorption of light by the water would probably limit photolysis to the upper 100 cm in most natural waters (7). Hydrolysis of anthracene has not been found to occur.

Microbial degradation of anthracene has been measured in short-term radiotracer experiments. A mixed culture of microbial strains derived from soil around an oil drilling site rapidly converted anthracene to polar compounds, with over 90% conversion within 90 min (3). In samples of sediment from an oil-contaminated stream, anthracene was readily broken down (half-life = 12 d), but only 10% of the radiotracer appeared in the polar fraction. In sediment from an uncontaminated stream, the degradation rate was an order of magnitude slower (2).

These basic studies on the fate of anthracene were all short-term, one week or less, measurements of single processes. They leave several questions unanswered: What is the fate of anthracene when the various transformation processes are acting simultaneously? What is the fate of anthracene in a system containing not one, but many, functional components of an aquatic ecosystem? What is the fate of anthracene and its transformation products over a longer period of time?

We have now examined the fate of anthracene over a 12-week period in laboratory microcosms modelled after pond ecosystems. These microcosms preserve the natural complexity of the decomposer and autotroph communities, and persist in stable condition for six months or longer (1). This paper describes the potential long-term fate of anthracene in an aquatic ecosystem with many of the biotic and abiotic transformation processes occurring simultaneously.

MATERIALS AND METHODS

MICROCOSM

The microcosms were initiated in January, 1977, using water, sediment, and organisms collected from a shallow pond. Water was collected in buckets. Sediment and masses of the dominant *Elodea* community were placed in tubs, where large debris was removed. Adiverse animal community was included in both the sediment and *Elodea* collections.

Two 80-liter slate-bottomed glass aquaria were acid-washed and placed in an environmental chamber. A 5-cm layer of sediment was added to each aquarium; a band of opaque tape prevented light from reaching the sediment from the sides. Pond water was then slowly siphoned into each aquarium through a home-made diffusion device to a depth of 30 cm. One 100-g (drained wet weight) portion of the *Elodea* community was placed in each microcosm; a few roots were pushed into the sediment to anchor the plants. The microcosms were covered with glass plates to reduce evaporation. The chamber temperature was initially set at 8°C (the ambient temperature of the pond), raised to 12°C in April, and to 16°C in May. A bank of cool white fluorescent lights provided 16,000 lux on a 12-h-light:12-h-dark cycle.

ANTHRACENE ADDITION

In mid-July, the microcosms were treated with [9-^{14}C] anthracene (specific activity = 32 μCi/μmole, Amersham/Searle). Approximately 1 μCi of anthracene was dissolved in 20 ml acetone and diluted to 500 ml with distilled water. The solution was divided into two 250-ml aliquots. One ml of each aliquot was withdrawn for radioassay, and the remainder was mixed with about one liter of microcosm water and slowly poured into the microcosm. The initial concentration of anthracene in the water was calculated to be 0.5 μg/liter.

SAMPLING AND RADIOASSAY

Two 1-ml water samples were removed from each microcosm two to five times weekly, placed in a scintillation vial with 10 ml Bray's solution, and counted on a Packard Tri-Carb Liquid Scintillation Spectrometer. Samples of sediment, *Elodea*, and filamentous algae were taken approximately once a week. Only the upper 1 cm of sediment was collected, except for the final samples in which entire cores were removed and sectioned. Samples of sediment and organisms were dried overnight at 55°C, weighed, oxidized with a Packard Tri-Carb Sample Oxidizer, and counted.

The final samples were taken 70-84 days after anthracene addition. In addition to the samples described above, periphyton from the aquarium walls, snails, snail eggs, zooplankton, and water mites were collected. The snails were removed from their shells, and all samples were then dried and oxidized as described above.

EXTRACTION AND THIN-LAYER CHROMATOGRAPHY

Water samples for extraction and thin-layer chromatography (TLC) were taken from the microcosms 11 times during the course of the experiment. Sediment samples for extraction were taken at the end of the experiment; only the upper 2 cm were used. Algae samples were removed and extracted on day 42. *Daphnia magna* were abundant at the time of anthracene addition, and 10-12 individuals were removed daily until the population was depleted.

Samples were extracted as follows: a) Water samples were acidified with a mineral acid and extracted twice with ethyl acetate in a 1:5 ratio of solvent to water. For each extraction, solvent-water mixtures were agitated for 45 min on a wrist-action shaker. b) Wet sediment samples were extracted overnight with acetone in a Soxhlet apparatus. Acetone was evaporated from the extracts, and the remaining liquid was extracted three times with equal volumes of ethyl acetate. c) Excess water was blotted from *Daphnia* and algae. Samples were weighed, homogenized in acetone, and centrifuged.

The extracts from each sample were pooled, dried over anhydrous calcium sulfate, centrifuged, and reduced to 2 ml under nitrogen. One ml of the extract was counted by liquid scintillation spectrometry to determine total extractable ^{14}C, and the remainder of the extract was subjected to thin-layer chromatography on silica gel 60 TLC plates. Radioautograms were prepared with X-ray no-screen film. Residues remaining after extraction of algae and sediment were oxidized to determine unextractable radioactivity.

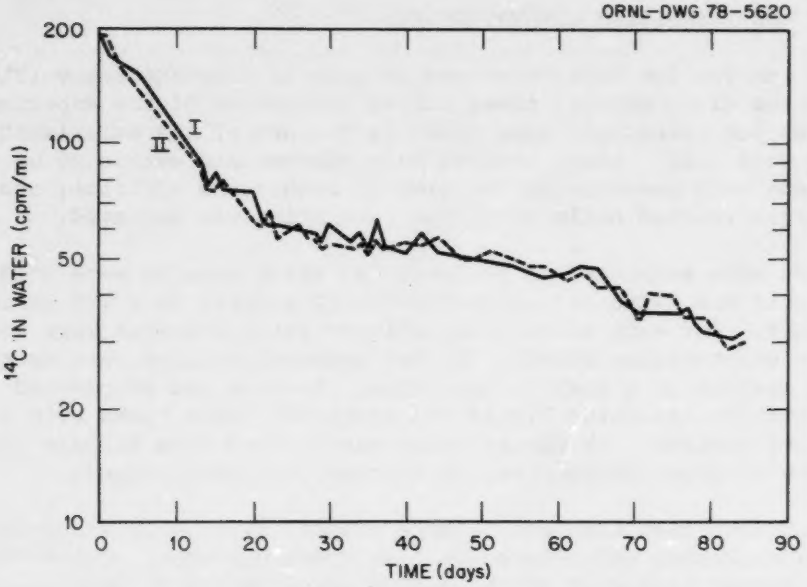
RESULTS AND DISCUSSION

DISTRIBUTION OF ^{14}C ACTIVITY

The ^{14}C activity in the water declined rapidly for the first 15 days, but more slowly for 50 days thereafter (Fig. 1). The mean first-order kinetic rate constant was 0.055 day^{-1} for days 0-15 and 0.007 day^{-1} for days 20-65. Rate constants determined for several metals in similar microcosms (unpublished data) ranged from 0.026 day^{-1} (selenium) to 0.23 day^{-1} (mercury). There was a suggestion of accelerated ^{14}C loss over the last 20 days of the experiment.

Table 1 shows the ^{14}C activity in all microcosm components 12 weeks after anthracene was added. Approximately 30 cpm/ml (15% of the initial ^{14}C) remained in the water. Most of the ^{14}C accumulated in the upper 2 cm of sediment, with much lower activity below this level. The total activity in the sediment was estimated to be 10.2×10^6 and 8.0×10^6 cpm for the two replicate microcosms. The recovery of label from the microcosm (92-114%) indicated that volatilization of ^{14}C compounds was negligible (Table 2).

All organisms accumulated activity to about 10^3 times the activity in the water. In each microcosm, highest activities were in water mites and snail tissue. These animals probably represent the ends of the grazing and detrital food chains, respectively, implying that anthracene or its degradation products may undergo biomagnification (accumulation up the food chain).



^{14}C Activity in Water: Anthracene Microcosms

Figure 1. ^{14}C activity (cpm/ml) in mocrocosm water. Solid and dotted lines represent averages of duplicate samples from replicates I and II, respectively.

TABLE 1. FINAL ^{14}C ACTIVITY IN MICROCOSM COMPONENTS TWELVE WEEKS AFTER ADDITION OF 10.4 x 10^6 cpm AS ANTHRACENE

Component	cpm/g Dry weight	
	Microcosm I	Microcosm II
Water[a]	31.6	30.5
Sediment		
Upper 2 cm	7,220	6,080
Lower 5 cm	875	374
Plants		
Elodea	25,800	28,000
Algae (benthic)	22,500	29,800
Periphyton	17,800	20,100
Animals		
Zooplankton	12,400	26,700
Water mites	31,900	45,700
Snails (tissue)	30,100	56,800
Snails (eggs)	21,300	21,600

[a] Water activity in cpm/ml.

TABLE 2. ^{14}C BUDGET

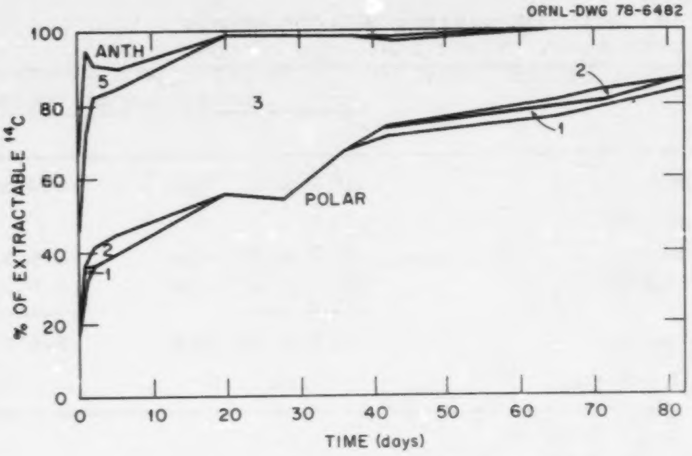
	Anthracene microcosms I	Anthracene microcosms II
Added	10.4×10^6 cpm	10.4×10^6 cpm
Recovered		
Water	1.7×10^6 cpm	1.6×10^6 cpm
Sediment	10.2×10^6 cpm	8.0×10^6 cpm
Total	11.9×10^6 cpm	9.6×10^6 cpm
% Recovered	114	92

DEGRADATION AND TRANSFORMATION OF ANTHRACENE

Chromatographic analysis of water, sediment, *Daphnia*, and algal extracts showed the presence of anthracene, four radiolabelled derivatives, and an unresolved polar fraction (Table 3). Co-chromatography of sample extracts with internal non-radioactive standards permitted identification of anthracene ($R_f = 0.84$); however, R_f's of 9-anthraldehyde ($R_f = 0.40$), anthraquinone ($R_f = 0.31$), and anthracene-9-carboxylic acid ($R_f = 0.01$) did not agree with R_f values of radioactive unknowns. The contribution of each compound to total extractable activity in the water during the experiment is shown in Figure 2. Only trace amounts of anthracene were recovered from the water after day 20. The disappearance of compound 5 closely followed the disappearance of anthracene, suggesting that anthracene was converted directly to compound 5, which was not persistent. Compound 3 was present in large quantity throughout the experiment. This compound constituted as much as 49% of the extractable radioactivity in the water (day 28), and although its relative abundance decreased thereafter, it was still present when the experiment was terminated on day 84. Forty-five percent of the total ^{14}C in the water was not extractable on day 84.

TABLE 3. PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN EXTRACTS

Compound	R_f	Day 6		Day 42		Day 84	
		Daphnia	Water	Algae	Water	Sediment	Water
Anthracene	0.84	22.3	9.4	1.5	0.9	32.1	--
5	0.76	19.3	5.6	1.6	0.8	15.7	--
4	0.34	--	--	--	--	--	--
3	0.27	22.7	39.5	40.5	24.7	29.5	13.1
2	0.08	--	4.9	--	--	--	--
1	0.04	--	--	1.9	2.0	6.7	2.4
Polar	0.00	35.7	40.6	54.5	71.7	16.0	84.5



Distribution of ^{14}C Activity in Extracts from Water

Figure 2. Distribution of ^{14}C activity in extracts from microcosm water. Compounds are numbered as in Table 3. Data are averages from replicate microcosms.

Distilled water, to which [9-^{14}C] anthracene was added, yielded anthracene, a polar fraction, the four derivatives found in microcosm extracts, and an additional compound with $R_f = 0.34$, after 7 days in the environmental chamber. The latter compound, which constituted 14% of the radioactivity extracted from the distilled water, was never detected in microcosm extracts. Figure 3 is a comparison of extractable fractions from microcosm water and from distilled water. After 7 days, the ^{14}C profile from distilled water was very similar to that of microcosm water on day 12. Anthracene was nearly absent from both systems. The large polar fraction recovered from distilled water (45%) as well as from microcosm water (48%), is particularly striking since it indicates that anthracene is readily degraded to polar compounds in the absence of microcosm biota. In studies of abiotic degradation of anthracene, Southworth (7) found that photolysis was rapid but hydrolytic breakdown was slow; therefore, the anthracene transformation products detected in the microcosm water and in the distilled water were probably due to photolysis.

Unextractable ^{14}C accounted for 40% of the activity in the upper 2 cm of sediment, and 30% of the activity in the lower sediment, after 84 d. At least part of this ^{14}C was probably incorporated into bacterial cells. One-third of the extractable activity, or 17% of the initial addition, was anthracene, and another large fraction was compound 5 (Table 3). The polar fraction was only 16% of the extractable activity. These findings are consistent with observations of Herbes and Schwall (2). The data imply that microorganisms in the sediment do not readily degrade anthracene, and that anthracene in the sediment could provide a source for continued exposure of benthic organisms to the parent compounts.

Daphnia were found to contain higher levels of extractable anthracene than was found in water (Table 3). The distribution of activity in extracts of algae was not markedly different from the distribution in water extracts taken at the same time, except that more of compound 3 was present (Table 3). Sixty-five percent of the ^{14}C in the algae was unextractable.

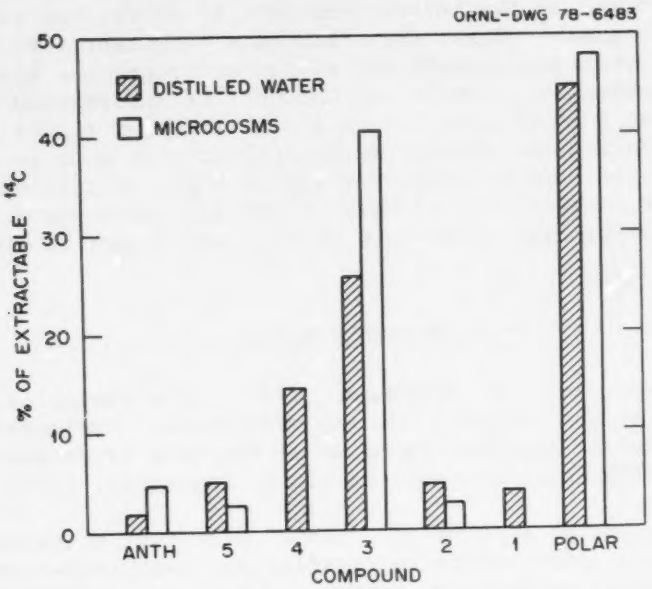


Figure 3. Comparison of ^{14}C distribution in extracts from microcosm water and distilled water. Distilled water was extracted 7 days after anthracene addition. Microcosm data for day 12 were interpolated from Fig. 2. Compounds are numbered as in Table 3.

CONCLUSIONS

Anthracene disappeared rapidly from the water. After 84 days, 17% of the anthracene initially added was recovered unchanged in the sediment. Approximately 40% was degraded to more polar compounds, apparently by photolysis. An additional 35% was unextractable from the sediment, probably reflecting incorporation into bacterial cells. Seven percent was transformed into an unextractable form in the water.

Several of the degradation products persisted in the water or the sediment for the duration of the experiment. The most abundant product (compound 3) was found in all microcosm components examined. Compound 4, one of the main degradation products formed in distilled water exposed to light, was never found in the microcosms, suggesting that it was broken down further by the microcosm biota.

Anthracene was much less susceptible to degradation when sorbed to sediment than when dissolved in water. Since sorption occurs rapidly in shallow water, a significant fraction of the anthracene entering a pond or stream would accumulate in the sediment. This would probably be available for uptake by organisms interacting with sediments (8).

Pond microcosm experiments can provide valuable information about the long-term environmental fate of an organic pollutant. In a microcosm, the pollutant is subjected to the interactive effects of many abiotic and biotic transformation processes. Thus, the microcosms represent a level of complexity between single-process experiments and actual environmental events, and provide one means (mechanistic models are another) of integrating simultaneous processes. Because pond microcosms are stable for many months (1), the persistence of degradation and transformation products as well as the parent compound can be assessed over extended periods of time. Microcosm experiments with PAH and other constituents of coal conversion effluents will provide a link between single-process laboratory studies and future studies in the field.

LITERATURE CITED

1. Giddings, J. M., and G. K. Eddlemon. 1977. Some ecological and experimental properties of complex aquatic microcosms. Presented at the Ecological Society of America Symposium on the Role of Microcosms in Ecological Research, East Lansing, Michigan, August 23, 1977.

2. Herbes, S. E., and L. R. Schwall. 1978. Microbial transformation of polycyclic aromatic hydrocarbons in pristine and petroleum-contaminated sediments. Presented at the ASTM Conference on Sediment Microbial Activity, January 30, 1978, Fort Lauderdale, Florida.

3. Herbes, S. E., L. R. Schwall, and G. A. Williams. 1977. Rate of microbial transformation of polycyclic aromatic hydrocarbons: a chromatographic quantification procedure. Appl. Environ. Microbiol. 32:244-246.

4. Herbes, S. E., G. R. Southworth, and C. W. Gehrs. 1976. Organic contaminants in aqueous coal conversion effluents: environmental consequences and research priorities, pp. 295-303. *In* D. D. Hemphill, ed., Trace substances in environmental health, vol. 10.

5. Leo, A. J. 1975. Calculation of partition coefficients useful in the evaluation of the relative hazards of various chemicals in the environment. *In* G. D. Veith and D. E. Konascwich, eds., Symposium on Structure-Activity Correlations in Studies of Toxicity and Bioconcentration with Aquatic Organisms. International Joint Commission, Burlington, Ontario.

6. Mackay, D., and W. Y. Shiu. 1977. Aqueous solubility of polynuclear aromatic hydrocarbons. J. Chem. Eng. Data 22:399-402.

7. Southworth, G. R. 1977. Transport and transformations of anthracene in natural waters: process rate studies. Presented at the ASTM 2nd Annual Symposium on Aquatic Toxicology, Cleveland, Ohio, October 1977.

8. Walter, M. T., and H. Johnson. 1977. A model system to study the desorption and biological availability of PCB in hydrosils, pp. 178-195. *In* F. L. Mayer and J. L. Hamelink, eds., Aquatic toxicology and hazard evaluation. ASTM STP 634.

DISCUSSION

BIODEGRADATION IN MICROCOSMS

MATSUMURA: Microcosms are like the Internal Revenue Service; whenever someone hears the word, they wince. They are difficult to define, but we must try to agree on something. In the past, people have confused microcosms with model ecosystems. We have conveniently defined a model ecosystem as something that we are trying to model containing more than one biological species. We are talking about microcosms in a much wider approach using any method which simulates natural events. Of course, there is little difference between bringing some of the "poison" into the laboratory to study the complex field events and taking something from nature and trying to study it simplified in a lab. For instance, when you attempt to study some compound underneath a fluorescent light to determine photodegradation, by our definitions, this is a microcosm. The idea is to simulate sunlight and study the compound's fate in this environment. Basically there are four microcosm approaches we can think of: 1) the physical-chemical approach, often times called "benchmark" approach, whereby scientists determine the chemical properties--lipid solubility, water solubility, volatility, etc., whatever physical-chemical character--which may affect the behavior of chemicals. 2) The second approach is a pure culture or simplistic type approach whereby one strain of microorganism or one tube of a photocell or whatever is utilized; this is not a complex system; you are studying only one reaction at a time. 3) The third approach is the model ecosystem type whereby you have more than one species and you are trying to study some complex parameter like accumulation. 4) The fourth approach is concerned with mathematical modeling. In this, scientists try to analyze the reactions from a systems point of view. Basically you can say that there is no difference in any scientific approach and anything you use to study field conditions in the laboratory is a microcosm. That is my point of view. Of course, like the blind man trying to assess what the elephant looks like, one is looking at only one side.

P. ROGERS: Dr. Clesceri, you talked of the validation of a model. Can you elaborate on what you feel validation of a model is? Addressing yourself to questions such as: what kind of sampling is reasonable before one can say that a model is verified? Would you expect if there were a major perturbation, in Lake George for instance, that a verified model, or your model, would be capable of predicting the results of this perturbation, because it changes an input? And should a model be tested with different lakes before being declared as verified?

CLESCERI: First of all, of course our model is good, and yes, I think that it could do a reasonably good job at predictions. How often should one go out to validate? That's really a hard question; as far as I'm concerned, the more the better. I think the real problem is the tremendous seasonal variation. With a seasonal type of sampling, validating four times a year is nowhere near enough. I think the sampling variability is too great and one has to do an intensive kind of sampling regime in order to get a good validation. That's my personal opinion, and perhaps I differ from other mathematical modelers. Because I have come up with some of the field work. Consequently, I feel quite wedded to the field approach. Your other question was, can the model apply to different lakes? I think so, yes, lakes of a similar class. I would feel that a model could be applied to oligotrophic, or nesotrophic or eutrophic lakes or what have you. But the point is, it would be to a class of lakes, and you would verify within that class.

WRIGHT: I would think you have the goal of subjecting the system to a more realistic perturbation. For example, the effects of a drought, or the effects of a period of heavy winds, a temperature change on the lake which leads to a change from diatom dominance to blue-green algae dominance. Is this your goal?

CLESCERI: Very definitely.

WRIGHT: Because these other perturbations are very unreal.

CLESCERI: The reason I brought these out was to show the usefulness with regard to this kind of experiment. It simply never could be done, and it's almost an academic exercise. The real usefulness of the model is to ask those realistic questions such as the ones you mentioned. Yes, clearly, that is our goal.

BAUGHMAN: I wonder if you have considered using models as an aid in arriving at decisions if one has results from the kinds of tests (biodegradability, microbial activity, etc.) that we've been talking about in this meeting. One of the things we have not addressed is: given results from these tests, how does one draw a conclusion? It seems fairly obvious that mathematical models can be used to aid in the interpretation of these data or to arrive at a decision of what should be done with a compound based on the kinds of effects that may result therefrom.

CLESCERI: This is where I think we all get cold feet.

BAUGHMAN: Well, somebody is going to have awfully hot feet. These are the hard decisions that have to be made. Can we use this approach to answer some of these questions?

CLESCERI: I would say, given the proper data, and building the model on solid data, yes! I would go so far as to say they can be, and should be, used for that kind of prediction. But what worries me, there are a lot of models that are not built on proper data, and under those conditions I think we have to be very careful.

FERGUSON: There is a point you brought up I want to reiterate. It is extremely important because a lot of management decisions are waiting for the appropriate model so managers can decide what to do and what not to do in terms of allowing corruption of the natural environment. What many of the more complex models are doing is trying to simulate a natural unimpacted environment. The great multitude of potential impacts precludes generating a model which is able to make predictions on more than one of these potential impacts. It's important to list the kinds of impacts that the model you are talking about is capable of making predictions on. For example, dumping in a particular toxic material is not going to be handled very readily in most of the models we are generating. I constructed a model of trophic dynamics in an eel grass bed. The problem was the possible effects of dredging on this bed. While we can say if you dredge the eel grass bed, then it's gone; the model wasn't really needed to make that kind of prediction. Another possibility is maybe the dredging caused some turbidity which reduced light penetration into the eel grass bed. The model we made didn't predict solar insulation of the grass bed, so we said, let's pretend the grass was only growing at half the rate. That is an example of the kinds of problems we have. We need to explain to government agencies that the models we develop are extremely limited in their perspective. That is a characteristic of models. It has to be designed to answer particular questions. At this point, we don't know which question is the most important to answer. There are so many possibilities that it's important when we do establish some sort of model, we make a list of the potential capabilities in prediction. I'm not sure where your model stands in that regard. I wonder if you could explain what kinds of perturbation your model could potentially make predictions about?

CLESCERI: I agree with everything you said. This model can handle any range of conditions for any one of those compartments. As Dr. Wright mentioned, changes that would happen due to a drought, could be predicted with this model. What would happen, for example, if there were a large dumping of phosphate or nitrogen or a sudden deficiency of oxygen? These could be handled by this model by simply varying those compartments. Obviously, there is a rate with each one of those changes which then would be coupled to the model. I would just like to mention, we are presently working on a pesticide model to be coupled to this model. Because of the solubility problems with toxic organic materials, the question of where the toxics are going is extremely important. So coupling with a pesticide model should produce that kind of information and then we can predict what the ecosystem response will be. First of all you have to know where the toxics are going, and the pesticide model will provide that.

SIKKA: Dr. Pritchard, I was wondering if you acidified and extracted the water phase with methylene chloride. I would guess the radioactivity in water is partially due to p-nitrophenol which would have gone into the organic phase had you acidified before extraction.

PRITCHARD: Yes, our water samples were extracted under both basic and acidic conditions, so I think we are getting all the *p*-nitrophenol out. I'm unsure as to the nature of the unextracted materials, but it could be pesticide bound to biological materials such as bacteria.

STERN: Incidentally, if I knew you were working on that first system we would have insisted on greater support for your laboratory.

PRITCHARD: If Tom Duke is here, I hope he takes note of that.

STERN: In the last slide, you indicated that your aged material in the flowing system was more active than in the fresh, and the converse is true in the cored system. It could be that in your cored system you get adsorption to biomass or solid materials. You may get adsorption to a level which might be inhibitory whereas in your continuous system this might not happen. So that what you are seeing is a localized concentration to inhibitory levels in your biomass and therefore a drop of viability of your cells.

PRITCHARD: We have seen very little inhibitory effects by methyl parathion except at very high concentrations (10 ppm) and I therefore feel toxic effects could not account for these differences. Also, since continuous flow experiments have not yet been performed with an intact core, it is difficult to answer your question. What we may be seeing is once you scoop out the sediment and water and put it into the continuous flow system, it may require an acclimation period before normal conditions are obtained. Thus, the faster rate might be the more indicative of a natural environmental condition.

STERN: The other point is that in a continuous flow you may need a certain adaptation period before you start to get things happening.

PRITCHARD: No, it seems that that was not the case and that's what surprises me. Some of our data indicate degradation occurs very rapidly once you start the experiment. I feel we need to take a close look at those initial rates. These may be the most reflective rates of what is actually going on in the environment. I would also like to make a comment regarding bacteria in both the coring and the flow-through systems. We have been unsuccessful in our attempts to isolate bacteria which will degrade methyl parathion, amino methyl parathion, or *p*-nitrophenol. We tried a variety of approaches but still have not picked up any sole carbon source utilizers. Likewise, in following the concentrations of bacteria in the water column and in the sediment, they show no trend that can be correlated with degradation processes.

LEE: I was interested in your comment about mixing vs a straight core. In our area of Georgia we work with a six-foot daily tide range. In such an area it seems mixed type sediments are more realistic than just taking a core, not allowing any mixing. So it depends on the area; certainly in our area we get mixing of several centimeters in a good tidal range.

PRITCHARD: Exactly, and my point is just that--there are a number of factors one should look at in terms of designing microcosms.

DRAGGAN: One of the usual justifications for using a mixed core rather than an intact entire core is that you overcome, in some measure, the complexity of heterogeneity of the environment which should increase the replicability within the experiment and enhance the ability to duplicate subsequent experiments. Do you find that your eco-cores are any more or less replicable? Is the confidence of the data any better? I think there is some evidence now that intact cores, like eco-core, can be just as replicable.

PRITCHARD: Yes, I agree. They are definitely replicable, and we've seen very good replication with our systems. If we run triplicate cores, for example, they look virtually identical. This replicability, I feel, is not due to a reduction in complexity.

CHOU: Have you looked into the fate of dimethyl thiophosphate? At UC-Davis, scientists found that dimethyl thiophosphate was not degraded in their parathion systems.

PRITCHARD: No, we haven't looked but you're right, it did not break down.

RAWLINS: Have you any data on the incorporation of C-14 into the cells of the microbes?

PRITCHARD: I think some of the radioactivity we are seeing in polar unextracted material would represent just that type of incorporation.

CHAPMAN: Let me say, Dr. Slater, what a stimulating talk I thought yours was. In comment, we have some observations I'd like to share in connection with 2,4-D utilizing organisms. In the course of isolating cultures of this kind, one initially establishes stable communities and ultimately determines that the communities co-exist because of fastidious 2,4-D degraders. One can then isolate 2,4-D degrading organisms by supplying them with appropriate nutrients, either amino acids or co-factors, and work with them in pure culture. Ribbons and I worked with pure cultures on the catabolic pathways of orcinol and the closely related molecule, resorcinol. We reported different metabolic pathways by which these compounds were degraded. It's interesting that acetate, which we identified as an orcinol metabolite, is the basis for the community existence you described. I would like to suggest that if you observe that your *Pseudomonas*, which is capable of utilizing orcinol, will also grow with resorcinol, then one can make predictions based on the metabolic pathways as to what might happen to the community structure. The metabolic pathway by which resorcinol is degraded does not yield as much acetate, and it is quite possible that by switching very simply from orcinol to resorcinol, the community structure will fall apart.

SLATER: Yes, in talking to Dr. Ribbons, he made the same good point. We have done none of the biochemical work with this system, and it's something we wish to do.

FLOODGATE: How far do you think all these pretty relationships you show in the fermenter really represent what does on in the sea? I always get a bit bothered about spatial relationships--how the little molecules manage to get from one "bug" to the other in a swirling mass of water? Does it all happen on the sides of the fermenter as it might happen on a beach? Or is this something which we can really project and say it is going on in the oceans right now?

SLATER: My fermenters are a swirling mass. There have been very few attempts to compare directly the type of community that we observe in these really very synthetic laboratory systems with what may actually be going on in the natural environment. My feeling is that these are probably valid comparisons. There is nothing essentially different between the type of enrichment you do in a continuous culture system and the type of enrichment you do when you put a compound into a particular natural habitat. I think the diazinon work, old work not done in a fermenter, is a good example. The original observation of the relationship between the *Arthrobacter* and *Streptomyces* was simply careful microbiology. Those two populations were enhanced over others when diazinon was applied to soil. Now we haven't done a reciprocal of trying a diazinon enrichment in a fermenter. You have very good evidence of the type of enrichment happening in soil, and if you can do it there, I am quite sure you could do it in a fermenter.

PRITCHARD: At what point are you going to draw a line in terms of accessing persistence in an enrichment like this? If you let the system incubate long enouch, a population may eventually evolve which will break it down. But is this evolutionary process environmentally realistic?

SLATER: Well, this refers to George Floodgate's point a little. I don't think we give enough consideration to one of the key points in microbial systems. That is, they are very versatile and very adaptive. In the dipicolinic experiments, clearly, there is some sort of adaptive process. We had to run the fermenter for 180 days, a considerable length of time. From a natural environment point of view, I think you've got to consider these points. These considerations are difficult in screening and testing programs but they can be done. Nevertheless, those sorts of activities are quite likely to occur in natural environments to some point. You then get what may appear to be essentially a non-degradable compound becoming a degradable compound. A much greater authority than myself is Professor Pat Clark in London. Her view is very much that we've been subjecting the environment to all sorts of indignities over the last 100 years with respect to antibiotics, etc. The tremendous potential is there to adapt and to involve the new systems, new mechanisms can deal with it. There is obviously the question of limitations at some point.

PRITCHARD: I've heard the criticism many times that in continuous-flow systems the useful organisms may wash out due to the selective nature of the system. You will quite often end up selecting for a population that is growing on a contaminant in your media. My question is, are you saying we might not have to worry about that as much?

SLATER: Well, I think you do have to worry. You may well be missing organisms. We've done a number of different compounds and it's really very impressive how very rapidly a complex soil system in these enrichments can change to a very few number of organisms. There are a number of things that you can do about it. You can continue to reinoculate for periods of time to see whether you can put back an organism which may fit into a simple enriched community which will achieve degradation of a compound which is not being degraded. So I think that is a real problem. There are other things we ought to begin thinking about. How can we develop continuous-flow systems so that we don't get this simplification? One of the things that Prof. A. Balkins is looking at is designing a system which allows the introduction of particulate matter in a homogeneous sense--not a colloidal but dispersed sand or clay particles or whatever. It seems probable that if you have an interface or some sort of increase in complexity you will sustain a great number of organisms in the community. So I think that the thing that you can do technically is to try to make sure that you get a better starting community.

PRITCHARD: Can I make just one more comment, relative to what you just said? I think it is really very critical at this point. We have found that methyl parathion is not degraded in a water only system, even when the influent water is providing a continuous inoculation. However, as soon as you add the sediment to the system, degradation commences.

SLATER: The one thing that worries me is the attitude that one must have a pure culture which is degrading this compound. I'm not saying that is your attitude, but I think a lot of people do have this attitude. We must consider corporate activities between organisms where you have genetic potential to a particular function split between different populations. Now, if I may be a little bit speculative, there is very little evidence apart from parathion and one or two others. My feeling is that those sorts of relationships are more common than we are prepared to accept at this moment, because of our conditioning to the way in which we do enrichments.

DiGERONIMO: A lot of times in the natural environment the introduction of xenobiotics may be episodic. Could you comment on enriching if the test compounds are going to disappear and appear at different times, not added on a continuous basis, but on an episodic one?

SLATER: The basis of this position is that you want a selection pressure of some sort so that you can get the adaptation processing. The problem is whether that kind of system would be continuous enough to force the organisms in the direction you want to be going. I don't really know. I don't think we've got any experimental evidence which would show whether or not that is a better or worse system for enriching. I think, however, from plasmid work which is perhaps related in a fringe way, that once you have the capability located in the population, even if you remove the selection, it will stay there and be in a relatively low level. So I don't know, it's an open question.

RAYMOND: What would happen if you did not "insult" your system with such high concentrations? Don't you think that probably leads to your limiting the number of types of microorganisms that survive in the system? Why only two or three? I noticed your concentrations were 0.5 g/liter or higher.

SLATER: Those are the concentrations you apply to the system, they are not necessarily the concentrations at which you conduct the enrichment. The value of a continuous-flow system is that you can conduct an enrichment at greatly reduced concentrations. If you recall my orcinol experiments, at low dilution rates, were exceedingly low. What is, I think, a criticism of what we have done is that we supplement at high nitrogen and phosphorus concentrations. That may not be relevant at all in a natural situation. The ratios could be variable and so it's just a matter of doing natural experiments. We chose higher concentrations since it provides a higher biomass so that we could do what we wanted to with the cells. But I think it is a point that should be taken into account when you are doing these community enrichments.

COLWELL: May I make a comment in regard to the need for mixed population studies? I should like to amplify the point you made about plasmids. I do think that plasmids play quite an important role in degradation in the environment. For example, we have found that in mercury-transforming microorganisms, 90% of the organisms may carry plasmids. We've been able to cure the bacteria of the plasmids and show that loss of mercury resistance or metabolism is associated with loss of the plasmid. Furthermore, we have examined drug resistance in *E. coli* and been able to transfer this characteristic to autochthonous *Vibrio* species in Chesapeake Bay. We're in the process now of working on the transfer of mercury metabolism amongst Pseudomonads. With this kind of genetic exchange there is a need to study mixed populations possible in natural systems. When examining degradation studies in a given system I will be very interested in Professor Balkins' results with his particulates, because I think that technique may create a population mass, insuring plasmid transfer. However, I would take issue with the statement that once the process is in the population it will stay there. Not quite so, because we have discovered that you can isolate acid and gas-producing *Pseudomonas aeruginosa* quite readily via an MPN test in a polluted area. Now, we all know that gas-producing *Ps. aeruginosa* are quite extraordinary. It is possible that the Pseudomonad picks up a lac gene via a plasmid and loses the plasmid quite readily. Thus the potential gene exchange may be quite significant but won't be quite as stable in situations as yours, accounting for erratic kinds of results observed for some systems.

SLATER: I would agree with that and my statement was an over-simplification. We have someone working in the lab on stability of drug-resistant plasmids, and we did some early work on the stability of organisms carrying lac operon, and they were unstable. Place the organisms into conditions of carbon limitation and they would fall apart. There was a reason for this, but the interesting thing is that with the natural plasmid, if I may use that word, of *E. coli* carrying certain drug resistances, they are exceedingly stable. We tried carbon and phosphorus limitations, and there are certain modifications but the plasmid, as such, stayed there. Ellwood's group also studied this and they found a similar sort of stability. Can I go back to the original point about the plasmids in the first place? Plasmids beg the question to an extent because they already contain the necessary genetic information for particular transformation in a particular pathway. The question is, where did they come from in the first place? My view is that mixed systems are the point at which various capabilities can be developed perhaps in separate organisms. What you may be seeing with plasmid mediated systems is the next step up where there is an aggregation of those activities into a cooperative state.

COLWELL: Sort of a community corporation?

SLATER: Yes, they put together a plasmid which can then be transferred around a population, and you get coordinate control. That is really speculative, but I think the point is they have to get the basic genetic information in the first place. It seems to me quite likely that the wider the genetic base in the community the more chance there is that various components can evolve particular capabilities to deal with a new compound, and then perhaps later put them together on a plasmid.

COLWELL: I quite agree, but I would just add one point. It's significant that we frequently find plasmids in *Pseudomonas*. Stability may well be a function of the plasmid moving amongst species of the genus, as well as amongst related genera.

LASKOWSKI: Dr. DePinto, I would like to add to your comment regarding role of models. I think a very good thing might happen in modeling. We'll probably arrive at a series or battery of models that will describe various physical situations. If one knows key physical and chemical properties of materials you can begin to get some idea of what might happen if those materials were placed in these different environments. I agree, I don't think there will be any one model that will serve this purpose, but it will be extremely useful to have a battery of models not only in the mathematical sense, but also in the physical sense, that these materials could be run through.

PRITCHARD: I have a comment relative to your use of the term chemostat. I've gotten now so that I shudder every time anybody mentions the word chemostat in a context like this. I think the explanation is very simple. A chemostat is a highly defined, very specific type of laboratory technique in which bacteria are growing as a function of a single limiting substrate under steady-state conditions. As soon as we begin to work with mixed cultures of any kind or get wall growth of any sort, or use multiple undefined substrates, then the term chemostat should not be used. The second thing about chemostats is that the growth of bacteria therein can be easily described with Monod kinetics. Many other types of continuous culture systems cannot be readily modeled with Monod kinetics.

DePINTO: I would like to make two comments on what you just said. First of all, if you survey recent literature, such as by Reeves, there are other mathematics describing use of chemostat that do not depend on Monod. In many cases these are being applied to uptake rates and decomposition rates in the modeling of phytoplankton in the Great Lakes area. They do not depend on Monod equations. Secondly, I recognize that there are limitations, certainly, of a flow-through system, but I think that a chemostat or flow-through system offers the ability not to duplicate but to simulate the natural system.

PRITCHARD: I'm not arguing that the continuous culture is not a great technique to use. Certainly, I wish more people would apply it, but I'm saying the use of the term chemostat has to be reserved for a very specific process.

DePINTO: Certainly, flow-through system is definitely better.

SLATER: Can I just back up what Hap said? Professor Pirt, who I think of all people is perhaps one of the few that can justifiably get involved in the semantics of the terminologies of continuous systems, suggested that the generic word to be used ought to be continuous-flow culture system. From that, you then derive a whole gang of different systems, chemostat being one, turbidistat, etc., there's a whole range of other sorts of systems. So the preferred term ought to be continuous-flow culture system.

Can I just make one point about modelling that worries me greatly? I have considerable doubt at this stage of development about the validity of some of these models. I'm not going to talk about attempting to model or simulate complex ecosystems. Let's just talk solely about continuous-flow culture systems where we may have a relatively simple community or a few cultures. From the attempts people have been making to model and explain in mathematical terms the various activities that are going on in a system, to describe substrate concentrations, population levels, and so on, it is transparently obvious that they fall short of reality. The point I would like to make is that we are trying to develop models without the fundamental understanding of the biological processes that are involved. For example, with the Monod kinetics there is no reason to suppose that the Ks value, saturation value, is growth rate independent. We always treat it as such, but there is some evidence that there may well be a variation in Ks as a function of growth. The way we apply it, we assume it's a constant. What I would advocate is, at the moment, we ought to concentrate far more on looking at the biological processes, try to work out how those factors are modified. The second point I would like to make relates to an argument that I have had with Dr. Bazin at the Queen Elizabeth College in London, who you might know does a lot of work on modelling of nitrification in soil columns. And this is that you can improve your model by just heaving in whole heaps of constants and eventually make it look like what you've got in nature, etc. Those constants have no biological basis whatsoever; so, if I had a preference, at this time, it would be to look much more closely at the biological relationship between population and the relationship with the physical environment before we go overboard on too many models.

DePINTO: Let me say first of all I'm not a modeller; I consider myself to be a microbiologist. I started using models in order to get more of a conceptual grasp of what is going on in the system and to try to assimilate data from a wide range of experimentalists. I think a model is a research tool and not necessarily an answer, at least for quite a long time.

BOLLAG: I would like to add some comments from a biological viewpoint. For me, it is difficult to understand how it is possible to use a model predicting the fate of an organic molecule if it is not yet known which kind of transformation can take place. It is my opinion that almost no xenobiotic exists from which all transformation possibilities are known. How is it possible to model the fate of a xenobiotic whose pathway has not been been determined?

DePINTO: In many ways I disagree with you, because I think it's never too premature to start developing a model to be used as a research tool in coordinating experimental data. Secondly, I think in meetings ten years ago, sponsored by the EPA, they were interested in modelling the fate of phosphorus and phytoplankton in lakes, one could hear some of the same kind of things you just stated. Ten years later, they have these models working for the Great Lakes, and they are being verified and improved upon for Lake Erie. They've found it a very desirable tool to be used for communication purposes.

LIU: We have about ten people who are interested in modelling. I find one very important thing: they should pay more attention to understanding the lake system. The system is very complicated; for example, the bacteria biomass distribution is not uniform, particularly in the lake sediment and under the interface. You have 10^8-10^9/ml bacteria less than 1/2 centimeter of surface, but in the lake water column only about 10^3-10^4/ml. The sediment or the surface is not stable. For example, on Lake Ontario, you have currents of ten knots per hour. Actually, lakes are constantly changing; the hypolimnion and epilimnion also change with the seasons. When you develop a model, you cannot use such data as total organic carbon because of so-called labile organic matter. What are they measuring, not all organic matter can be utilized?

BAUGHMAN: I find myself in an awkward position coming to the support of the modellers, because I certainly don't consider myself a mathematical modeller. It seems to me the chief complaint against models is that they don't always accurately predict behavior in some environment that we are interested in. I find little difference between use of a mathematical model as a tool or guide to our research and decision making as a basis for complaint than perhaps the fact that Dr. Bollag did not use some *Bacillus* instead of his fungi. The model has a purpose it is intended to serve and certainly will not serve a different purpose very well. There is a point that should be made here; models can serve at least two purposes in the context that we talk about. One is to simulate some actual environment, like Lake George or Pensacola Bay or some aspects of these. The kind of model that was referred to here is one that is intended to simulate the kinds of things that can happen rather than to predict exactly what does happen in some specific site. It appears to me that one of the things we are badly missing is some basis for establishing what is important. For example, we have seen that films occur on water surfaces and organisms are concentrated there, that if we have sediments in a system, if they are mixed or non-mixed, you get different results. My question is, are those differences important and what is the basis for telling if they are important? If the fact that we can simply see something different is to be the basis for importance, then we have an infinite problem on our hands. I would suggest that our models won't improve and, perhaps, some of our bases for interpretation won't get any better, unless we start to address this question.

BAUGHMAN: Dr. Giddings, I have a couple of questions. What kind of lights were used in your study? Were the tanks usually covered? And what was the depth of the water?

GIDDINGS: Cool white fluorescent, the depth was 30 cm, and the tanks were covered. There was light in the sediment.

BAUGHMAN: I'm curious why you expect photochemical degradation of anthracene, because anthracene absorbs fairly low in the ultraviolet region. Cool white light is very short for anthracene, particularly if you covered your tank with plastic. You would expect photochemical degradation of anthracene to be trivial unless there is significant sensitized degradation, which you should not get in the distilled water system.

GIDDINGS: I can't answer that except that is what we found.

BAUGHMAN: I was wondering, did you attempt to look at the ratio of anthracene in the sediments to the ratio in the water during the course of these experiments?

GIDDINGS: We weren't successful in taking sediment samples until near the end of the experiment so we don't have that data.

BAUGHMAN: One would fully expect anthracene to sorb to the sediment, thereby drastically reducing the rates of processes like volatilization and photochemical degradation in those systems.

GIDDINGS: Yes, that's what we thought.

DAVIS: I happened to hear you talk about the photocatalytic activity. There is something working against all of this. Photocatalytic activity is indeed occurring out there in these estuaries, especially highly turbid Texas estuaries, that is a protective mechanism, when you have a secchi disk reading in ours, for example, Florida estuaries are beautiful by comparison, you know. Secchi disks of about four to six inches normally with high silt loads from the rivers and precipitation or sedimentation later. Before long these things settle out and build up, and such things as the oyster accumulate these compounds. And I think it is important what you pointed out. What was the salinity of your microcosms? Were they entirely fresh water?

GIDDINGS: Yes, this was spring water.

DAVIS: Do you have any data on conductance?

GIDDINGS: About 100 microohms initially but it goes down after that.

DAVIS: From what I've found, not on anthracene but on related compounds, as you go up into 3% estuarine salt levels you can remove a factor of 10 in the degradation rates. In other words, with the same number of "bugs" per milliliter you don't get degradation of the same amount per day.

GIDDINGS: Thank you.

JOHNSON: I have a question. How do you plan to use this microcosm, what will you do with your data?

GIDDINGS: Our initial objectives are to see how it works. Initially we plan to run a number of the representative PAH's as I pointed out, through the microcosms, see if we detect the same sort of trends that we saw in simpler test systems.

JOHNSON: Assuming that you are successful, how would you report these data to a federal agency?

GIDDINGS: I'm not sure. The microcosms task group has been beating this around quite a bit. Regulations we are talking about from here seem mostly to require some sort of standardized test or battery of standardized tests. These microcosms, I've pointed out, aren't standardized and aren't standarizable. The real world is often not standardized or standardizable.

JOHNSON: Would you feel this would be representative of a community or something of this sort?

GIDDINGS: Yes.

JOHNSON: I am a microbiologist, but since I do represent a laboratory that is concerned with fish I was wondering how could you exclude fish from your pond?

GIDDINGS: Well, that was one of the concessions we made to the laboratory conditions. We've tried including fish in the microcosms but this was not a degradation study; rather, what effect did they have on the ecology of the microcosms? They have less of an effect than I would have expected considering the fish density is much greater than you would expect in a real pond. We found increased rates of production in the presence of fish. We found that bluegill or fathead minnow would eat up snails and that would have additional secondary and tertiary effects on the ecology of the pond. But we haven't done a degradation experiment to see what effects that would have.

JOHNSON: You made a statement rather in passing that fish or animals were not an important sink for the compounds. I was wondering how you could make that statement.

GIDDINGS: Well, I'm speaking in terms of the distribution of the compound and its derivatives at the end of the experiment. Little was incorporated into biological tissue, unless we count the 35% that was bound to the sediment. What was in the plants and animals was an insignificant fraction from a point of view of where the anthracene went.

JOHNSON: But you also made a statement, that after 84 days you knew something about this compound in regard to what would happen in a stream or pond. And actually what you would know is what would happen in 84 days in this little microcosm that excluded fish and other animals, and probably other invertebrates also.

GIDDINGS: Perhaps, we haven't sampled the pond. The way to answer you would be to compare what happens to anthracene in a microcosm with what happens to anthracene in a pond. That latter study isn't practical. About all we can do is to compare the ecology of the microcosm to the ecology of the pond, the water chemistry of the microcosm to the water chemistry of the pond. We find great similarity between them. I think that the microcosm environment is very similar to the pond environment. The ultimate test of a model, an LC_{50} test, or a microcosm test is whether that really does apply to the world. That's one of the big problems we've got to work on.

COONEY: On the question of light effects, I think there was a comment from a gentleman over here who implied that the chromophore of anthracene might not be long enough to account for the photochemistry. I think that one does not have to rely upon a UV-absorbing chromophore because when white light is incident upon an aquatic system, you get sufficient singlet oxygen generated so that it can be lethal to large numbers of microbes. There is a recent report indicating that in estuaries there is sufficient singlet oxygen present to account for transformation of organic pollutants to a measurable degree.

PRITCHARD: Could you get the same results in a smaller system?

GIDDINGS: Well, we've experimented with smaller systems and they have certain advantages: they're easier to work with; they're easier to set up; they require less material; you can put more of them in a smaller space; you don't need as much anthracene; you don't need as much C-14. But the difficulties we found with smaller microcosms were that they are less stable, took longer to equilibrate, were less replicable, and basically were harder to sample repeatedly without causing strange effects in controls and things like that. They were not amenable to repeated sampling the way that we need to sample.

GARNAS: In listening to your discussion on your system, I failed to see the main purpose why you used the system. Had you been looking at fate of a chemical due to photochemistry, hydrolysis, or sorption, I would tend to believe you would study those properties in your laboratory environment while trying to simulate the real world. You've taken the substrate from a pond, you've eliminated a certain trophic level, and put them in a glass box in your laboratory. You really haven't defined to us where the real world is in that system.

GIDDINGS: I would submit that the microcosm is an ecosystem similar in function and structure to a pond ecosystem with the exception of fish. We can debate how important fish are to microbial degradation. We've also found that the microcosms are similar to the ponds from which they're constructed. But, I don't think that's essential to accepting them as a valid ecosystem which is much more amenable to experimentation than a real pond is. George Southworth and Steve Hervies have generated these data on single processes and have tried to put it all together into a model that predicts what's going to happen to anthracene when all these processes are interacting at once like in nature. Except for some qualitative statements like, "photolysis is likely to be important," and "microbial degradation is likely to be important," they weren't able to say what the results of all these things were going to be. One approach is to just continue to modify your model, refine your model, stick in as many mechanisms to try to come up with a prediction of the natural fate of anthracene. We've shortcut that by treating the microcosm as a black box. We're using a microcosm instead of a mathematical model to integrate these processes. They are all happening simultaneously, physically, not mathematically.

GARNAS: But, it would appear that just from the fate of the chemicals, you don't really have a good grasp as to what is happening. Is it photochemical; is there indirect evidence that there is or is not volatility; is it a biodegradation process or some other process? The difficulty that you've run into now is that the complexity is so great that you can't zero in on any individual component or individual process.

GIDDINGS: That's right. For instance, the experiment we did with the distilled water made it much easier to understand what was happening in the microcosm water. I use that as an example to show that we have to interact with the lower levels of testing to understand what is happening to the microcosm. The microcosm shows us some things that we could not have predicted, or could not have been sure of, by putting together the results of single process studies. For instance, the competition between sorption and photolysis on the fate of anthracene in the water, you can try to get rates defined real carefully, and then see what happens when you have them running simultaneously. Does absorption beat out photolysis? You can try to do it mathematically, but you can do it just as simply in the actual biological system.

CONTRIBUTED PAPERS

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RESISTANCE OF POLLUTANTS TO DEGRADATION IN SALINE ENVIRONMENTS

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ABSTRACT

Organic compounds which usually degrade rapidly in fresh water environments were shown to become almost refractory in saline wastewaters. Salt concentrations were maintained at or near that of estuarine environments. Additionally, saline wastewater BOD data can be questioned if the test is conducted in accordance with the method currently being employed. Microbial populations were found to be the reason for as much as a 30% difference in BOD results with tests conducted at different salinities. Salt-tolerant bacteria, used as seed organisms in BOD testing, showed that BOD results were similar at 0%, 1%, and 3% salt concentrations when using standard (nonsaline) dilution water. Acclimated sewage seed produced BOD values significantly lower when saline wastes were tested. Equal concentrations of bacteria in the BOD test did not reduce equal amounts of organic materials when exposed to various salt concentrations. Sewage seed degraded 44 mg/liter/d organic material at 0% salt, 38 mg/liter/d at 1% salt, and 22 mg/liter/d at 3% salt. Bacterial genera which predominated in 3% salt were *Pseudomonas*, *Bacillus*, *Staphylococcus*, and *Flavobacterium*, three of the four genera being potentially pathogenic.

Hypersaline wastewaters also may not be amenable to conventional treatment methods. Activated sludge methodology applied to an organic amine wastewater containing 3.5% salt showed only 48% BOD removal and 52% TOC removal at an F:M loading rate of 0.2-0.5 lb BOD/d/lb MLSS. Settleability was seriously affected by increased salt concentrations. The SVI of most units can be expected to range from 0.4 ml/g despite high bacteria densities of $3x10^6$/ml. Extended aeration is more efficient but

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requires detention times of up to 80 d. Application of nitrification methodology following activated sludge treatment of the hypersaline wastewater was necessary. Ammonia increased from 13 mg/liter to 70 mg/liter in the effluent. Ammonia removal averaged 65% in nitrification units with some sludge recycle. Anaerobic digestion of hypersaline wastewaters is an alternative provided the salt content is maintained below 1.3%. At a detention time of 15 d, anaerobic efficiency increased with decreases in salinity.

INTRODUCTION

Complications occur naturally in saline aquatic environments in the overall degradation of organic pollutants. The microbial population present in either estuaries or hypersaline industrial wastewaters may not function to their full capacity in degrading the organics for several reasons. The percentage of bacteria representing the most functional group for degradation purposes may not be the dominant microbiota in a heterotrophic population (10). Additionally, even relatively small amounts of heavy metals can reduce organic degradation rates (11). Temperature of the ecosystem corresponding to season of the year plays an extremely important role (2). Specific research investigations have demonstrated the complexity of functional metabolism of microbial populations in respiration and concurrent degradation of pollutants. Biochemical oxygen demand (BOD) testing has been used to evaluate waste loading and for design bases for waste treatment units. Yet, only recently has it appeared that that test (9) may not show the full waste loading or its presence in a saline environment.

In one study (5) the soluble waste portion in a salt water carrier was metabolized at the same rate by the microbial population in domestic sewage. However, the insoluble portion produced different rates, with that of domestic sewage in fresh water being three times greater than that of domestic sewage in a salt water carrier. It was further shown that only the degradation of suspended organic material was inhibited by salt and the dissolved organic fraction of the BOD was unaffected (8). Even salt-tolerant bacteria function in BOD removal rates according to the salt concentrations (3). Increases in BOD resulted when the salt level was decreased. Ten-day BOD's on salt water wastes were 3 to 6 times higher with standard dilution water than with a dilution water having the same salinity as the waste. Further, the rate of biological oxidation, K, may vary considerably under different test conditions (4). Wastewaters containing less than 10,000 mg/liter chloride diluted with fresh water were shown to have a K value greater than that of fresh water waste. The K value increased to the point equivalent to 50% of that of sea water. When the 50% level was exceeded, K decreased, until in 100% sea water, it was lower than in fresh water. This led to the hypothesis that a low salinity may stimulate microbial activity and result in increased BOD values, and in some instances the organic pollutants may persist because cellular constituents of lytic activity are preferred as a nutrient source to the organic substrate which has been introduced as a pollutant (6, 7).

This study was undertaken to further identify and quantify some of the reasons for reduced degradability of organic pollutants in marine environments and to ascertain whether the BOD test truly represents the organic pollution load in saline ecosystems. Microbial populations were examined for their relative efficiency of wastewater treatment in wastewaters having salinities comparable to those of the marine environment.

MATERIALS AND METHODS

The BOD tests reported herein were conducted in accordance with methodology listed in Standard Methods (9) except for those results obtained by manometric analyses (Hach Chemical Co. BOD Apparatus). Bench-scale activated sludge units were used for testing of the industrial waste (3). Nitrification and anaerobic digestion units additionally were bench-scale in size. Microbial genera in the wastewater and in the various testing units were identified by the methods outlined in *Diagnostic Microbiology* (1). The principal organic fraction of the industrial hypersaline wastewater tested in this research was an ethylamine complex. The wastewater had a salt concentration of 3.7%, a total bacterial population of 2.2×10^2/ml, and a BOD of 30 mg/liter, using the existing population in the wastewater as seed. Salt-tolerant bacterial seed for activated sludge units comprised Bacterium T-52 and *Paracoccus halodenitrificans* (3).

RESULTS AND DISCUSSION

Quantification of the effect of sodium chloride concentrations on a sewage bacteria-seeded glucose standard are shown in Figure 1. Also shown is the representative daily BOD for a 600 mg/liter sodium acetate solution. The data show dramatically how two salt concentrations, which approach the salinity of sea water, affect the results of a 5-d BOD. Those data were determined by the manometric technique. Using the salt-tolerant bacterial *Paracoccus halodenitrificans* (Fig. 2), similar effects were observed at the 5-d period, except for more rapid respiration rates in the early periods. The implication of the data shown in Figure 1 is that BOD results of saline wastewater should be viewed with caution regarding the total waste load, and may not be representative of the total organic concentration. Stimulation of salt-tolerant bacterial species using nonsaline BOD dilution water is indicated in Figure 2, especially for the acetate standard. Additionally, the survival of all sewage seed bacterial genera in saline environments is not assured in BOD testing. A noteworthy example of that is presented in Table 1. Declines in populations even in nonsaline BOD's to 5 d is indicated. More importantly, genera which survive at 3% salt concentration, similar to marine situations, are illustrated in Series V of that table. Potentially pathogenic genera, e.g., *Pseudomonas* and *Staphylococcus*, appear to have survived best. Distinct changes occurred in sewage seed bacteria populations during their acclimation to the salt concentrations and to the organic glucose standard. The raw sewage seed had an initial bacterial population of 10^7/ml. During the acclimation period to the glucose standard population levels dropped to 10^6/ml. Acclimation to the glucose standard containing 1% and 3% salt resulted in a bacterial population of 10^4/ml and 10^3/ml, respectively. This behavior resulted in lower

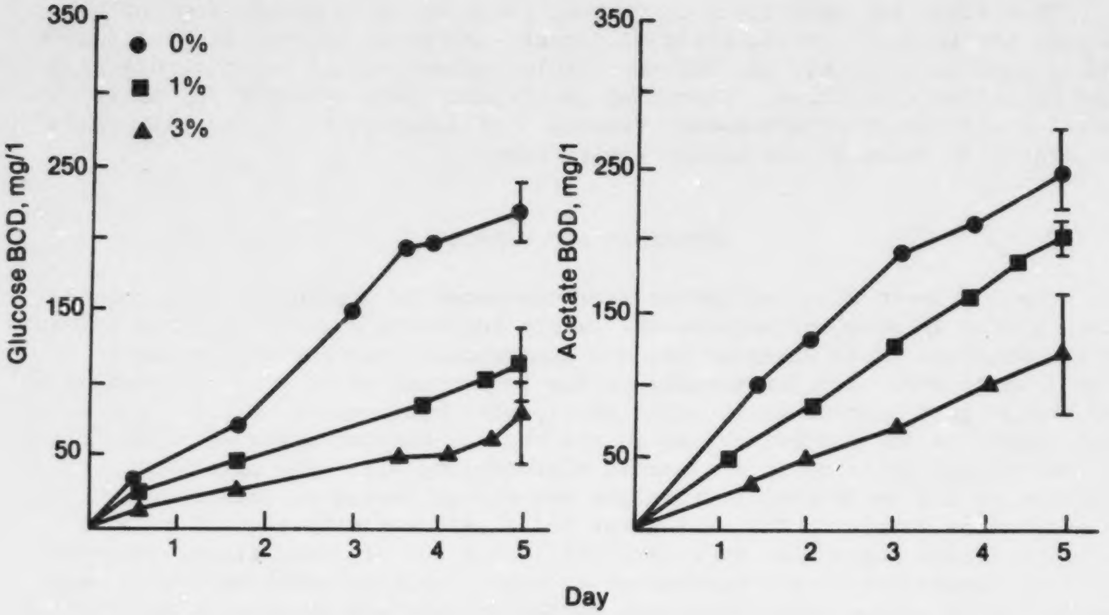

Figure 1. BOD values for glucose-glutamic acid and sodium acetate standards in three salt concentrations using sewage bacteria seed.

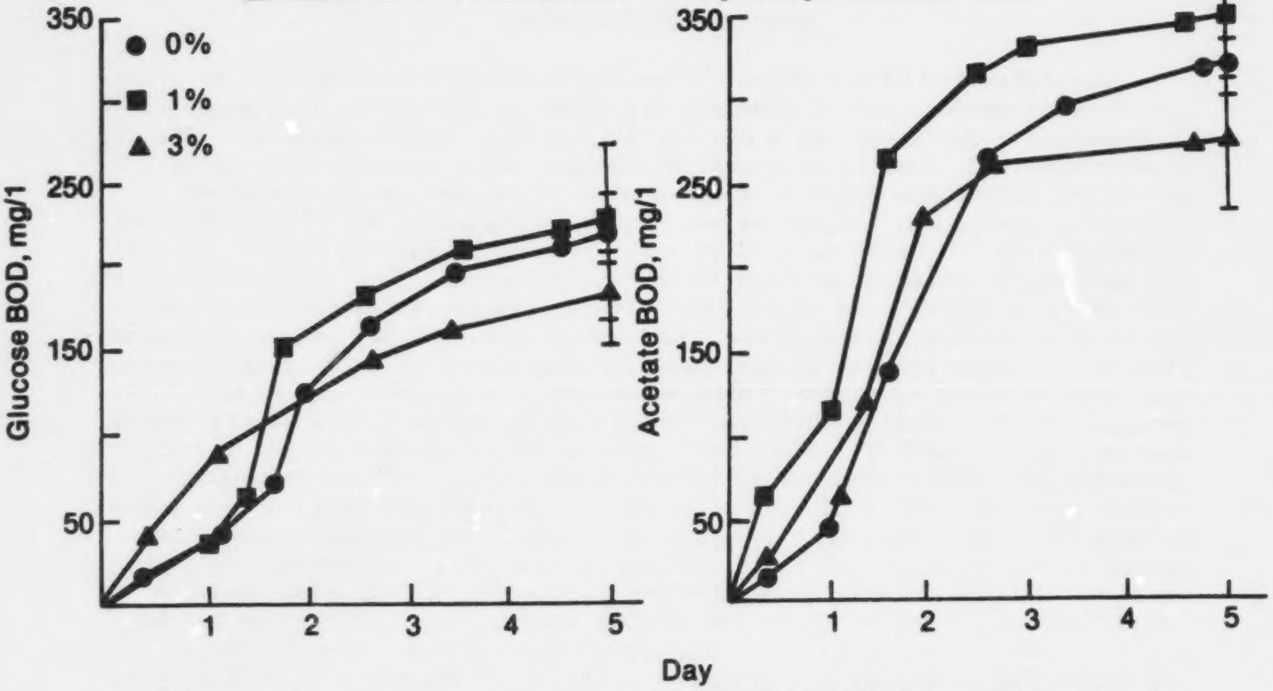

Figure 2. BOD values for glucose-glutamic acid and sodium acetate standards in three salt concentrations using *Paracoccus halodenitrificans* as seeds.

TABLE 1. DISTRIBUTION OF SEWAGE SEED BACTERIA IN GLUCOSE STANDARD BOD TEST AT DIFFERENT SALT CONCENTRATIONS

Genus	Series and days									
	Series I		Series II		Series III		Series IV		Series V	
	0	5	0	5	0	5	0	5	0	5
Pseudomonas	15%	77%	70%	40%	8%	13%	70%	71%	8%	14%
Bacillus	8%	7%	10%	30%	7%	35%	10%	11%	7%	16%
Staphylococcus	12%	2%	5%	--	7%	--	5%	--	7%	20%
Flavobacterium	--	--	10%	30%	7%	46%	10%	7%	7%	--
Unidentified *Diplococcus*	--	--	--	--	70%	6%	--	--	70%	44%
Acinetobacter	6%	--	5%	--	--	--	5%	11%	--	--
Streptococcus	4%	5%	--	--	1%	--	--	--	1%	6%
Klebsiella	10%	5%	--	--	--	--	--	--	--	--
E. coli	8%	2%	--	--	--	--	--	--	--	--
Alcaligenes	1%	2%	--	--	--	--	--	--	--	--
Neisseriae	8%	--	--	--	--	--	--	--	--	--
Serratia	8%	--	--	--	--	--	--	--	--	--
Shigella	6%	--	--	--	--	--	--	--	--	--
Salmonella	6%	--	--	--	--	--	--	--	--	--
Enterobacter	4%	--	--	--	--	--	--	--	--	--
Citrobacter	4%	--	--	--	--	--	--	--	--	--
Total No./ml	3.6×10^4	8.5×10^8	6.5×10^3	1×10^7	6.6×10^2	2.3×10^7	6.5×10^3	5.0×10^6	6.6×10^2	3.0×10^5
BOD	--	220	--	235	--	246	--	194	--	170

Series	Glucose Standard Salt Conc., %	Dilution Water Salt Conc., %
I	0	0
II	1	0
III	3	0
IV	1	1
V	3	3

initial populations in the BOD's conducted at 1% and 3% salt. During BOD testing, bacterial populations of the sewage seed increased from 10^2-10^3 in the saline diluted organic standard and increased 10^4- 10^5 during the same time period in the organic standards using standard (nonsaline) dilution water. The greater increase in the standard dilution water accounted for the increase in BOD values. Linear regression analysis was applied to the data for the glucose standard using sewage seed and the two salt concentrations comparing bacteria counts versus 5-d BOD values. Since the initial bacteria counts varied at the beginning of each BOD test, all initial counts were based at zero and the log increase in bacterial counts were regressed against BOD values. The linear regression of those log increases in bacteria on the resultant BOD values yielded a significant "r" value of 0.87 (Fig. 3). The "r" value is the linear regression analysis correlation factor and for the number of data analyzed to be significant at the 0.05 level, the "r" value must have been 0.49. At the 0.01 level it must have been 0.62. This explains some of the differences in the BOD values for saline wastewaters diluted with standard and saline dilution waters. Even with low initial bacteria counts, population increases in saline organic standards using the standard dilution water caused the BOD values to be higher than those for the same saline organic standard of the same salinity as that of the waste, which had a smaller population increase.

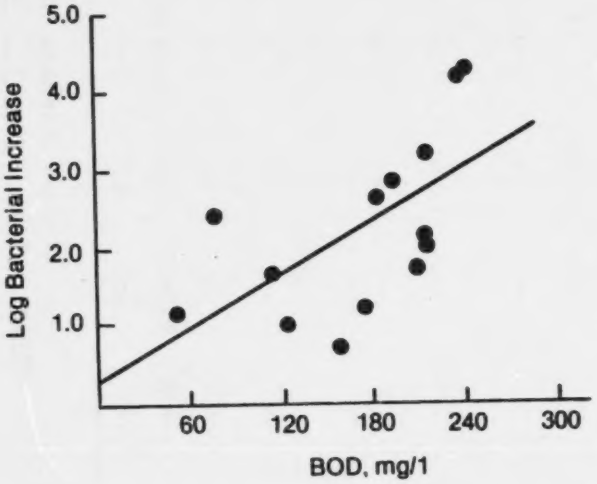

Figure 3. Sewage seed population increases in the 5-d glucose BOD's using various salt concentrations.

Average bacterial increases in the manometric BOD flasks and the corresponding BOD removals were regressed against time to determine if the same number of sewage bacteria utilized the same aount of glucose at different salt levels. The slope of those regression lines yielded the bacterial increase per day and the BOD removal per day (Fig. 4). With the same increase in bacterial population, the BOD removal rate was 44.4 mg/liter/d at 0% salt, 38.6 mg/liter/d at 1% salt and only 22.8 mg/liter/d at the 3% salt level. Sewage seed acclimated to the saline glucose standard resulted not only in low initial counts and slower growth rates but also in a slower utilization rate of organic material.

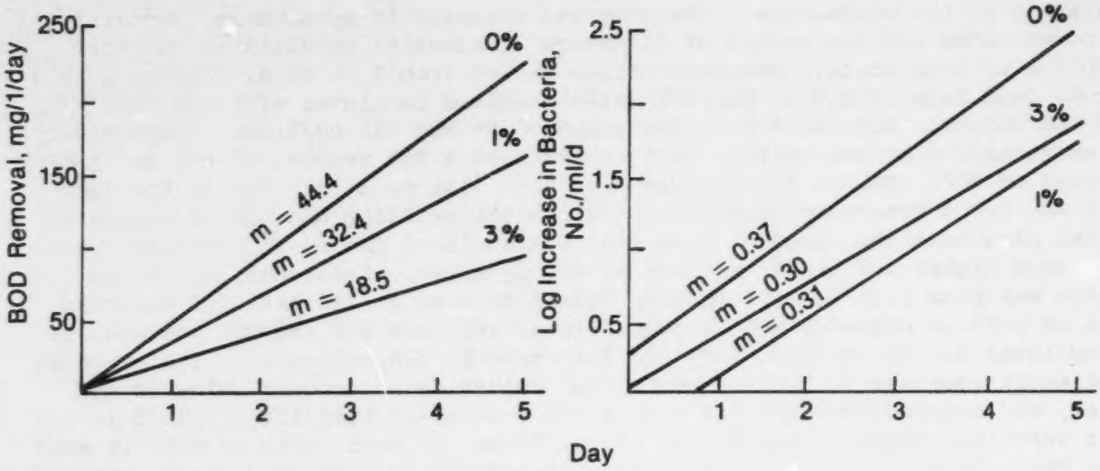

Figure 4. Slopes of BOD removal and bacterial population increases in manometric BOD tests using glucose and sewage seed at three salt concentrations.

Activated sludge treatment, comprising extended aeration, was applied to the industrial wastewater for different loading rates and detention times.

Bench-scale activated sludge units were used for monitoring the sedimentation, clarification, and organic removal in the industrial wastewater. Each unit had a capacity of 5 liters and an aeration rate of 0.0033 lb $O_2/ft^3/h$. The seed culture was a mixture of Bacterium T-52 and *Paracoccus halodentrificans*. The units were fed conventional loading rates (F:M) of 0.2 and 0.5 lb BOD/d/lb MLSS. After 60 d of treatment, feed rates were changed to yield an F:M ratio of 1.5 and 0.06. Total suspended solids (TSS) and volatile suspended solids (VSS) and the sludge volume index (SVI) were monitored to determine the amount and settleability of the sludge. Bacterial populations were monitored in the units to determine concentrations present at the different feed rates and if they were being flushed from the units. Total organic carbon (TOC) and BOD were measured to determine the efficiency of organic removal at the different feed rates. The BOD of the industrial wastewater was conducted using standard dilution water since our previous experimentation with the standard dilution water and a saline dilution water of equivalent salinity yielded equivalent values.

Analysis of the activated sludge unit treatment data through day 60 showed essentially no difference in BOD or TOC removal for the two conventional F:M loadings of 0.5 and 0.2 lb BOD/d/lb MLSS. Effluent BOD and TOC values for both units averaged 75 and 100 mg/liter, respectively, at those feed rates.

This represented a 48% removal of BOD and a 52% removal of TOC. Total suspended solids (TSS) remained high in both effluents, averaging 115 mg/liter. MLSS in the units ranged from 160 to 200 mg/liter. Settleability was poor with the Sludge Volume Index (SVI) ranging from 0.4 to 2.8 ml/g. Microscopic examination of the effluent showed a fine pinpoint type floc which remained suspended in the wastewater. The floc was granular in appearance, contained no bound water and was devoid of filaments. Bacterial populations averaged $3x10^6$/ml in both units. Detention times varied from 8 to 20 d. Changing to a higher feed rate of 1.5 lb BOD/d/lb MLSS resulted in higher effluent BOD, TOC, and TSS values. BOD and TOC values averaged 86 and 122 mg/liter, respectively, after reseeding of the units. This represented a 36% removal of BOD and a 42% removal of TOC. TSS in the effluent was high (121 mg/liter) due to the fact that the 2-3 d detection time did not allow the settling chamber to remove all of the slow settling sludge. Most floc was still of the small pinpoint type. MLSS were higher and ranged as high as 400 mg/liter. Settleability of the solids was poor with the SVI ranging from 2 to 4 mg/g. An extended aeration rate of 0.06 lb BOD/d/lb MLSS produced lower effluent BOD and TOC averages of 45 mg/liter and 85 mg/liter (68% and 58% removal, respectively). The pinpoint floc still remained in the effluent. TSS values never dropped below 60 mg/liter, and averaged 70 mg/liter with a SVI average of 1 mg/liter. MLSS in the unit were low, ranging from 100 to 120 mg/liter. A feed ratio of 0.06 lb BOD/d/lb MLSS yielded the highest quality effluent of the four feed rates examined. This feed rate is not practical for an activated sludge unit because of the long detention times which ranged from 60 to 80 d. The conventional feed rates of 0.2 and 0.5 lb BOD/d/lb MLSS produced similar effluent qualities. The higher feed rate (0.5) would be preferred due to the shorter detention times of 7 to 10 d. The rapid feed rate with a 2-3 d detention time yielded a poor quality effluent that was high in BOD, TOC, and TSS. Of the four feed rates, 0.5 lb BOD/d/lb MLSS yielded the highest quality effluent for the least amount of time. Due to the characteristics of this particular wastewater, a problem was encountered which made the activated sludge system ineffective as a single stage treatment method. Ammonia values increased from 13 mg/liter in the raw wastewater to an average of 70 mg/liter in the effluent from the units at conventional feed rates. Ammonia-nitrogen from the ethylamine complexes is an end product of carbonaceous degradation.

Removal of ammonia-nitrogen from the effluent of the activated sludge units by complete-mix nitrification reactors averaged 65% regardless of detention time and feed rates (Fig. 5). Ammonia concentrations were reduced from an average of 70 mg/liter NH_3-N to 25 mg/liter NH_3-N. Denitrification was started in the units but was lost due to the system setup which had no recycle of sludge. Denitrification bacteria were flushed from the units within 20 d. Nitrate-nitrogen averaged 49 mg/liter in the influent and 75 mg/liter in the effluent. BOD values were reduced in the units from an average of 73 mg/liter to 66 mg/liter and TOC was reduced from an average of 100 to 90 mg/liter. The plug-flow unit with sludge recycle removed 60% of the ammonia from the wastewater at a detention time of 15 d. The ammonia concentration decreased from an average of 75 to 30 mg/liter NH_3-N. Denitrification was accomplished but the removal efficiency was low, averaging only 25%. BOD values were reduced from 86 to 78 mg/liter through the unit and TOC was reduced from 122 to 106 mg/liter. Floc particles in these units would settle well and the TSS average was about 55 mg/liter.

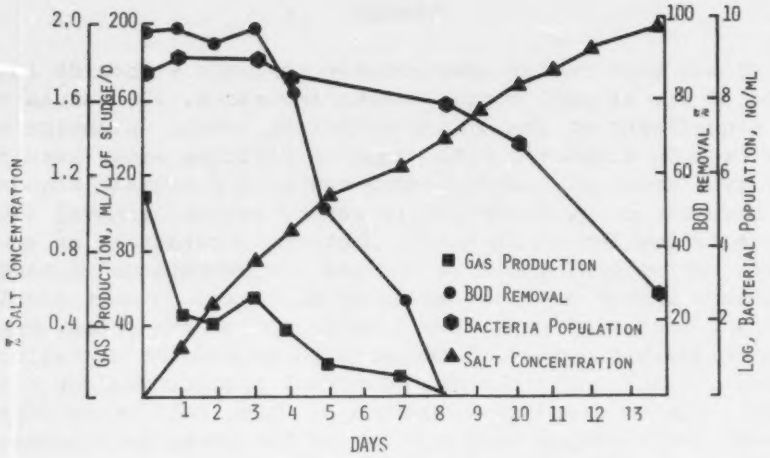

Figure 5. Nitrification capability for a saline wastewater at four detention times.

Observations during the operation of the anaerobic digesters showed a decrease in gas production and BOD removal with an increase in salt concentration (Fig. 6). When a salt concentration of 1.3% was reached, gas production ceased. At this time, there was only a small decrease in bacterial populations, therefore a salt concentration of 1.3% was inhibitive against one particular group of organisms, the gas producers. The bacterial population of the digester sludge was 4x10^{2}/ml when the salt concentration reached 2%. This indicates that anaerobic digestion may not be feasible for hypersaline wastewaters.

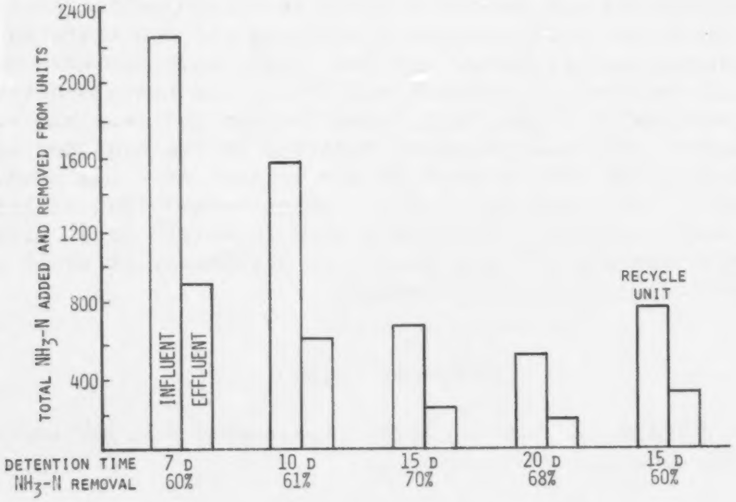

Figure 6. Anaerobic digestion performance with increases in salt concentration.

SUMMARY

The BOD values obtained in sewage-seeded organic standards showed a significant trend, i.e., as salt concentration increased, BOD values decreased. Changes were significant at the 0.05 statistical level. Dilution of sewage-seeded saline organic standards with standard dilution water resulted in BOD values higher than those of corresponding non-saline organic standards due to increases in bacteria populations and increased organic removal (BOD values) in the presence of low levels of salt. Bacteria populations of sewage seed correlated with corresponding BOD values and concentrations of salt in organic standard solutions showed that the addition of 1% and 3% salt resulted in a decreased initial bacterial population, decreased growth rates, and a decreased ability of an equivalent number of bacteria to degrade an equivalent amount of organic material. Each salt-tolerant bacterial species yielded comparable BOD values for each organic standard conducted at three salt concentrations. Population increases and K values were similar at the three salt concentrations and when using either the standard dilution water or the saline dilution water. Since sewage seed does not compare favorably with salt-tolerant bacteria in determining the BOD of saline wastewaters, for determination of the BOD of a wastewater with elevated or varying salt concentrations, a salt-tolerant bacterial seed should be used to insure an accurate and reproducible BOD value.

Bench-scale, continuous feed activated sludge units were capable of 48% BOD removal and 52% TOC removal at conventional feed rates using selected salt-tolerant bacteria. The best BOD and TOC removal occurred with the extended aeration process but the 60 to 80 d detention times are not feasible for activated sludge units. The feed rate of 0.5 lb BOD/d/lb MLSS yielded the highest quality effluent with a detention time of 8 and 10 d that would be acceptable for activated sludge units. A fine pinpoint floc with poor settling characteristics was noted at all feed rates utilized. This pinpoint floc may be related more to the characteristics of the bacteria in the saline media than to feed rate. Nitrification and denitrification in a hypersaline wastewater can be accomplished utilizing salt-tolerant nitrifying and denitrifying bacteria. While not as efficient as freshwater systems, those bacteria were capable of removing 60-70% of the ammonia-nitrogen and 25% of the nitrate-nitrogen from the hypersaline wastewater. Anaerobic digestion was not feasible with the hypersaline wastewater. The gas-producing bacteria in the digester sludge did not acclimate to the 3.5% salt content of the wastewater. Gas production ceased at 1.3% salt. This was the only treatment method that utilizes fresh water organisms and attempted to acclimate them to salt. If a culture of salt-tolerant anaerobic bacteria could be found, or developed, it would seem that this treatment method would also be feasible.

LITERATURE CITED

1. Bailey, R. W., and E. G. Scott. 1974. Diagnostic microbiology. C. V. Mosby Co., St. Louis.

2. Carney, J. R., and R. R. Colwell. 1976. Heterotrophic utilization of glucose and glutamate in an estuary: effect of season and nutrient load. Appl. Environ. Microbiol. 31:227-233.

3. Davis, E. M., J. K. Petros, and E. L. Powers. 1977. Organic biodegradation in hypersaline wastewaters. Indust. Wastes 23:22-25.

4. Gotaas, H. B. 1949. Effects of seawater on the biochemical oxidation of sewage. Sewage Works J. 21:818.

5. Kessick, M. A., and K. L. Manchen. 1976. Salt water domestic waste treatment. J. Wat. Pollut. Control Fed. 48:2131-2136.

6. Kincannon, D. F., and A. F. Gaudy. 1966. Sequential substrate removal after change in salt concentration. Biotech. Eng. 8:371.

7. Kincannon, D. F., and A. F. Gaudy. 1966. Some effects of high salt concentrations on activated sludge. J. Wat. Pollut. Control Fed. 38: 1148-1159.

8. Manchen, L. K. 1974. Unit operations in saltwater sewage treatment. M.S. Thesis, Rice University.

9. American Public Health Association. 1976. Standard methods for the examination of water and wastewater, 14th ed. American Public Health Association, Washington, D.C.

10. Walker, J. D., and R. R. Colwell. 1976. Measuring the potential activity of hydrocarbon-degrading bacteria. Appl. Environ. Microbiol. 31:189-196.

11. Walker, J. D., and R. R. Colwell. 1976. Oil, mercury and bacterial interactions. Environ. Sci. Technol. 10:1145-1147.

METHANOGENIC BIODEGRADATION OF AROMATIC COMPOUNDS

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ABSTRACT

Decomposition of a range of ligno-aromatic compounds to methane under strict anaerobic conditions was observed over varying periods of time. Compounds examined include vanillin, ferulic acid, cinnamic acid, protocatechuic acid, catechol, phenol, and syringealdehyde. Evidence for ring cleavage was provided by gas analysis and mass balance calculations, with conversion of substrate carbon to gas ranging from 60% to 98%. Methanogenic cultures maintained on ferulic acid comprise several species forming an anaerobic food chain. Inhibition of methane formation by bromoethanesulfonic acid (BESA) did not appear to affect decomposition of ferulic or propionic acids. Gas chromatography demonstrated the temporary buildup of acetate and proprionate as intermediates in methane production. Cultures inhibited with BESA also contained butyrate, isobutyrate and isovalerate, suggesting that pathways of decomposition may be different from those of benzoate. The resulting products of fermentation agree with the calculated stoichiometry, e.g., ferulic acid:
$C_{10}H_{10}O_4 + 5.5\ H_2O \rightarrow 4.75\ CO_2 + 5.25\ CH_4$.

Methanogenesis plays a key role in the carbon cycle by aiding mineralization of organic matter in highly anaerobic environments. Compounds which are not degraded in the water column may eventually find their way into marine sediments, most of which are anaerobic below the first few centimeters. The fate of ligno-aromatic compounds in such environments is of concern because they are pollutants discharged with wood pulping wastes, and may constitute a carbon sink if they are refractory under anaerobic conditions. This report provides evidence that a variety of lignin-associated aromatic compounds are biodegradable to methane under anaerobic conditions.

Lignin is a complex three-dimensional aromatic polymer consisting largely of phenylpropane building blocks (Fig. 1). Its biodegradation in any environment is considered to be a slow process (1). When the complex structure of lignin is broken down by physical, chemical, or biological action (15) a variety of aromatic compounds is yielded. For example, the alkaline heat treatment of lignin, as practiced by the pulp and paper industry, is expected to release a range of simple aromatics including those shown in Figure 1.

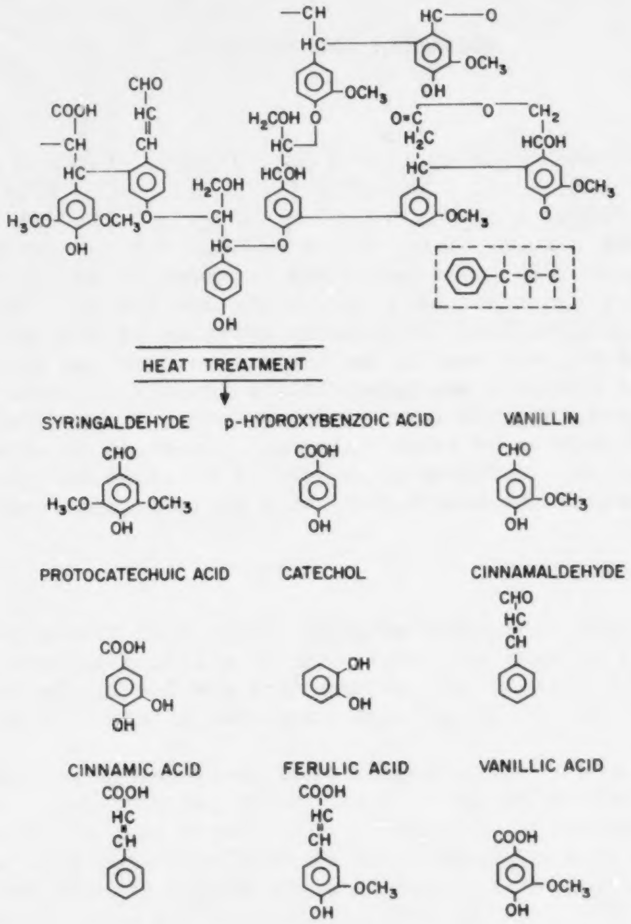

Figure 1. Model lignin polymer and compounds resulting from alkaline heat treatment.

The fate of these compounds in anaerobic environments is unclear. Extensive studies on aerobic metabolism of aromatic compounds (7, 14, 5) have taken place, while relatively little attention has been given to their anaerobic fate. Early evidence suggested that some simple aromatic compounds were fermentable to CO_2 and CH_4 (17, 4, 3, 10). Other investigators isolated individual species which anaerobically degraded aromatic compounds by photometabolism (16, 11, 6) and nitrate respiration (18, 19). A stabilized consortium of three predominant microorganisms also has been found to degrade benzoate to methane (9). Biochemical pathways proposed for the different kinds of anaerobic benzoate metabolism vary in the types of aliphatic acids formed (8). The manner in which these acids are further metabolized depends upon the particular organism or organisms involved. Our report focuses on the characteristics of the methanogenic fermentation of a model lignin derivative, ferulic acid, by an anaerobic microbial food chain.

MATERIALS AND METHODS

MEDIA

Pre-reduced defined media contained (per liter): 0.001 g resazurin, 0.04 g $(NH_4)_2PO_4$, 0.2 g NH_4Cl, 1.8g $MgCl_2 \cdot 6H_2O$, 1.3 g KCl, 0.02 g $MnCl_2 \cdot 4H_2O$, 0.03 g $CoCl_2 \cdot 6H_2O$, 0.0057 g H_3BO_3, 0.0027 g $CaCl_2 \cdot 2H_2O$, 0.0025 g $Na_2MoO_4 \cdot 2H_2O$, 0.0021 g $ZnCl_2$, 0.368 g $FeCl_2 \cdot 4H_2O$, 2.64 g $NaHCO_3$, 0.5 g $Na_2S \cdot 9H_2O$, and 1% (v/v) vitamin solution (20). The media was buffered at pH 7.0 by a bicarbonate/CO_2 system with a gas atmosphere of 30% CO_2 and 70% N_2. Oxygen was removed from the media by coiling followed by addition of the sodium sulfide reducing agent. Resazurin was used as an oxidation-reduction indicator. A C:N:P molar ratio of 100:15:1 was provided for aromatic substrate concentrations of 300 mg/l used in serum bottle enrichments. Pre-reduced replacement media, used for maintenance of stock cultures, contained an aromatic substrate concentration of 10 g/l. Additional amounts of nitrogen and phosphorus thus were required to sustain the same C:N:P ratio as in defined media.

SERUM BOTTLE ENRICHMENT

Defined media were inoculated with 10% (v/v) seed from a laboratory anaerobic digester fed primary settled sewage on a 15-d detention time. Serum bottles (250 ml) were flushed with oxygen-free gas for 20 min before addition of the inoculated media. Cultures were incubated in the dark at 35°C.

Syringes were used to add aromatic substrate, remove portions of the culture for analyses, and to monitor the volume of gas produced. Substrate concentration was determined by diluting a centrifuged sample and assaying it in a spectrophotometer at a characteristic UV wavelength for each particular aromatic compound. Gas composition was determined on a Fischer-Hamilton gas partitioner. Gas production and substrate concentration were corrected for background levels by subtracting values measured in a control culture devoid of aromatic substrate.

Cross Acclimations

Aliquots (7.5 ml) of acclimated stock culture were transferred to Hungate tubes, previously flushed for 10 min with oxygen-free gas. These cultures had not produced gas for several days before the transfer. Then 2.5 ml of different pre-reduced aromatic substrates was added to each tube, returning the 10-ml culture to original enrichment nutrient levels. A control tube contained no aromatic substrate. The onset, rate, and extent of gas production in the tube with the particular substrate to which the stock culture was acclimated was compared with gas production in the other tubes.

STOCK CULTURE MAINTENANCE

Acclimated stock cultures were maintained in serum bottles by regular replacement of 1/5 of the culture volume with fresh pre-reduced media. This replacement was made after the culture finished converting its previous supply of substrate to gas. After a 150-ml culture was shaken into a homogeneous mixture, 30 ml were withdrawn with a syringe and either wasted or transferred as inoculum to another serum bottle. An appropriate volume of replacement media was then added, with the balance of 30 ml being defined media.

INHIBITED CULTURES

2-Bromoethanesulfonic acid (BESA) was used to inhibit methane production in experimental cultures. BESA, dissolved in pre-reduced defined media, was added to cultures at the time of media replacement in a concentration of 10^{-4}M.

GAS CHROMATOGRAPHY

Samples for gas chromatography of volatile fatty acids (C_1-C_7) were prepared by acidifying a 2-ml portion of culture fluid with 2 drops of concentrated sulfuric acid and extracting this portion with 1 ml of ethyl ether. One µl of this ether extract was injected into a glass column packed with 10% SP-1000/1% H_3PO_4 on 100/120 chromosorb W AW (Supelco, Inc.). A hydrogen flame ionization detector was used in a Tracor MT 220 gas chromatograph.

RESULTS

ENRICHMENT CULTURES

Initial enrichment studies indicated that a range of aromatic compounds are degradable under strict anaerobic conditions (Table 1). Decomposition in enrichments was slow, first requiring an acclimation period of about 2 weeks, followed by gas production over an additional 2- to 4-week period. Cultures acclimated relatively easily to syringic acid and syringaldehyde in 5 d compared to 27 d for the more refractory catechol. The conversion of substrate carbon to gas was observed to vary, ranging from 59% with catechol to 98% with syringealdehyde. In most instances, more than 80% of the carbon was converted to gas, indicative that ring cleavage occurred under these strict anaerobic conditions.

When additional substrate was delivered to the same enrichment cultures, degradation to methane and carbon dioxide occurred without a lag. This is illustrated by the temporal relationship of phenol degradation (Fig. 2). Initial phenol enrichments required a 16-d lag (Table 1), while subsequent enrichments showed a relatively short 1-d lag. The conversion of substrate carbon to gas in Figure 2 is very rapid as evidenced by the coincidence of substrate disappearance and gas production. Methane is produced continuously throughout the entire gas-producing period and constitutes more than half the total gas produced.

TABLE 1. DEGRADATION OF AROMATIC COMPOUNDS UNDER STRICT ANAEROBIC CONDITIONS

Substrate	Acclimation lag (d)	Period of gas production (d)	% Conversion of substrate carbon to gas
Vanillin	11	29	86
Vanillic acid	13	27	86
Ferulic acid	13	28	83
Cinnamic acid	14	34	80
Benzoic acid	11	21	84
Catechol	27	12	59
Protocatechuic acid	15	17	74
Phenol	16	21	82
p-Hydroxybenzoic acid	15	17	65
Syringic acid	5	14	97
Syringealdehyde	5	15	98

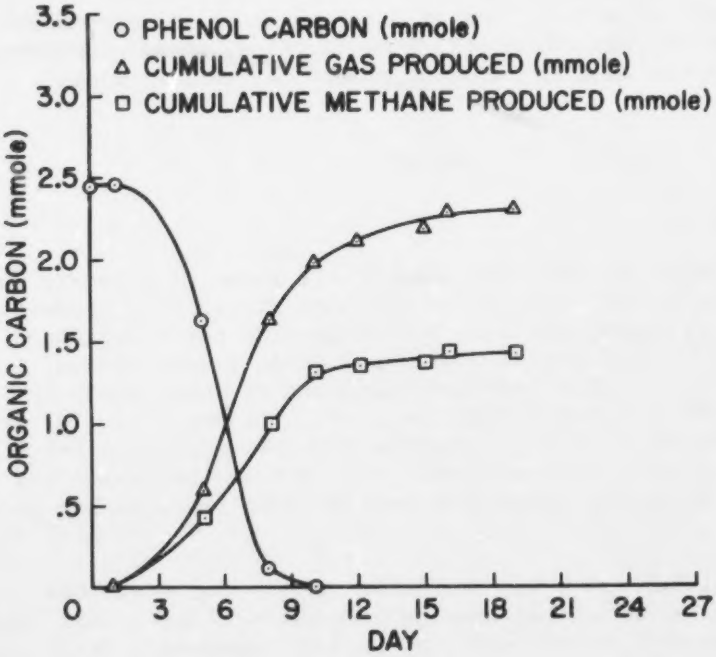

Figure 2. Phenol mass balance. Conversion of phenol carbon to CO_2 and CH_4.

The stoichiometry for the conversion of several ring compounds to gas is shown in Figure 3. As illustrated by these equations, 50 to 60% of the organic carbon can be converted to methane. Based upon these stoichiometry, the experimental conversion of these compounds to methane is summarized in Table 2. The amount of methane produced closely agrees with theoretical values (95-100%); while total gas values ranged from 77 to 94% of theoretical.

PHENOL $C_6H_6O + 4H_2O \rightarrow 2.5CO_2 + 3.5CH_4$

CATECHOL $C_6H_6O_2 + 3.5H_2O \rightarrow 2.75CO_2 + 3.25CH_4$

VANILLIC ACID $C_8H_8O_4 + 4H_2O \rightarrow 4CO_2 + 4CH_4$

FERULIC ACID $C_{10}H_{10}O_4 + 5.5H_2O \rightarrow 4.75CO_2 + 5.25CH_4$

Figure 3. Mass balance stoichiometry for four example aromatic compounds.

TABLE 2. CARBON BALANCES

Substrate	Total gas produced (mmole)		Methane produced (mmole)	
	Actual	Theoretical	Actual	Theoretical
Phenol	2.29	2.44	1.43	1.42
Catechol	1.76-2.67	2.87	1.21-1.89	1.55
Vanillic acid	1.73	2.03	1.02	1.02
Ferulic acid	10.7	13.7	7.01	7.18

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Cross acclimation studies were undertaken by feeding a culture previously grown on one aromatic carbon source a new and different aromatic substrate. Of the range of compounds tested, generally those with similar structure were readily utilized and converted to gaseous end products. Figure 4 is an example illustrating that a ferulic acid enrichment does not use phenol, but readily utilizes cinnamic acid resulting in immediate gas production. It should be noted that without this prior acclimation to ferulic acid, growth on cinnamic acid would require a 14-d lag period (Table 1). Cultures grown on ferulic acid were observed to readily utilize cinnamic acid, vanillic acid, vanillin, and acetate in cross acclimation studies (Fig. 5). As illustrated in Figure 5, the structure of a compound such as cinnamic acid may be similar enough that the organisms can readily utilize it. Immediate uptake of vanillin and vanillic acid, however, suggest that these compounds may serve as intermediates in decomposition of ferulic acid. This is supported by the observation that cultures acclimated to vanillin or vanillic acid cannot simultaneously utilize ferulic acid.

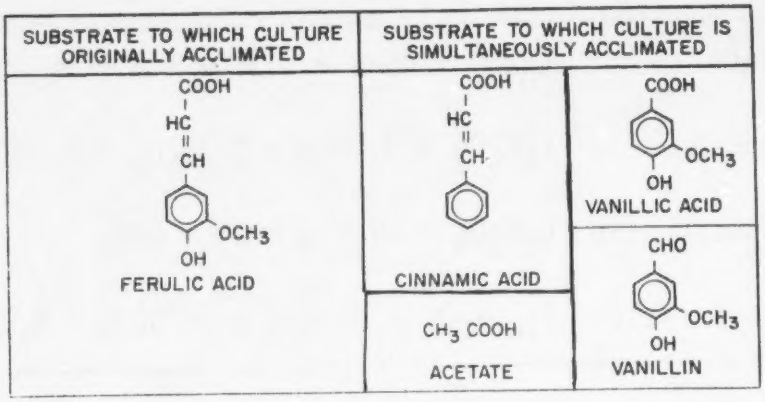
SUBSTRATE TO WHICH CULTURE ORIGINALLY ACCLIMATED
SUBSTRATE TO WHICH CULTURE IS SIMULTANEOUSLY ACCLIMATED
COOH
HC
CH
OCH3
OH
FERULIC ACID
COOH
HC
CH
CINNAMIC ACID
CH3 COOH
ACETATE
COOH
OCH3
OH
VANILLIC ACID
CHO
OCH3
OH
VANILLIN

Figure 4. Gas production in cross acclimation studies for ferulic acid acclimated studies.

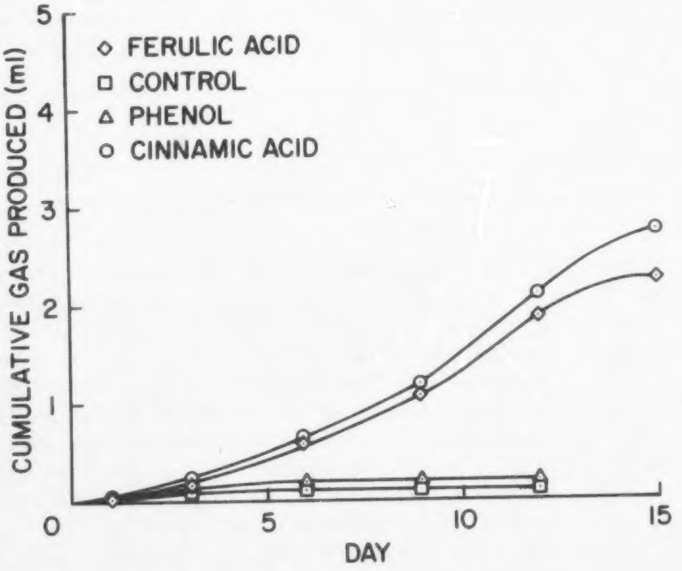

Figure 5. Cross acclimation results with ferulic acid cultures.

Since acetate is a known precursor for methane formation, and it is observed to be readily utilized by the ferulic acid culture, its presence as an intermediate in ferulic acid degradation was further examined by gas chromatography. Figure 6 illustrates the conversion of ferulic acid to gaseous end products with a temporary buildup of acetate. Significant acetate levels first appear after most of the ferulic acid has been degraded and gas production has begun. This is followed by the disappearance of acetate as gas production ceases. These results are similar to the observations on the methanogenic degradation of benzoic acid (9).

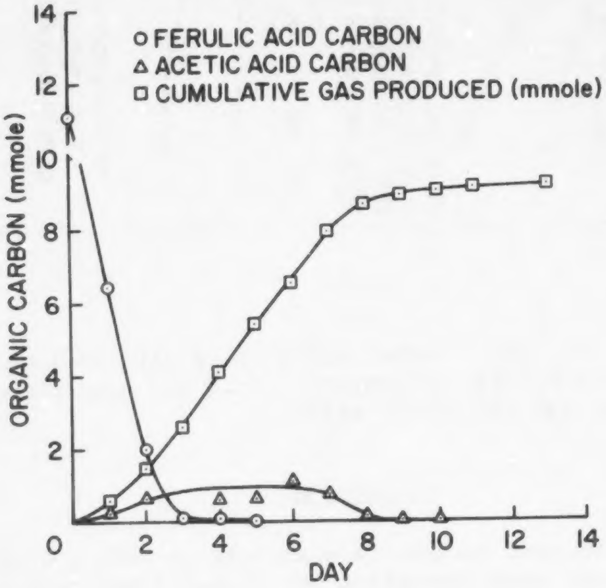

Figure 6. Ferulic acid conversion to methane with temporary appearance of acetate.

Detection of other volatile acid intermediates was thought to be hampered by their presence in very low concentrations. To enhance the buildup of these intermediates, active enrichments were inhibited with BESA, an analogue of coenzyme M (personal communication, R. Wolfe). It acts specifically by blocking methyl transfer in the conversion of acetate to CO_2 and CH_4. As shown in Figure 7A, ferulic acid is rapidly degraded; however, total gas production is less than 20% of that in an uninhibited culture. In addition, acetate concentrations were elevated to concentrations almost 4 times higher than that in uninhibited systems. Four other compounds, propionic, isobutyric, butyric, and isovaleric acid were detectable only in cultures inhibted with BESA (Fig. 7B), suggesting that when methane formation is not blocked, rapid conversion of these organic acids takes place preventing their detection.

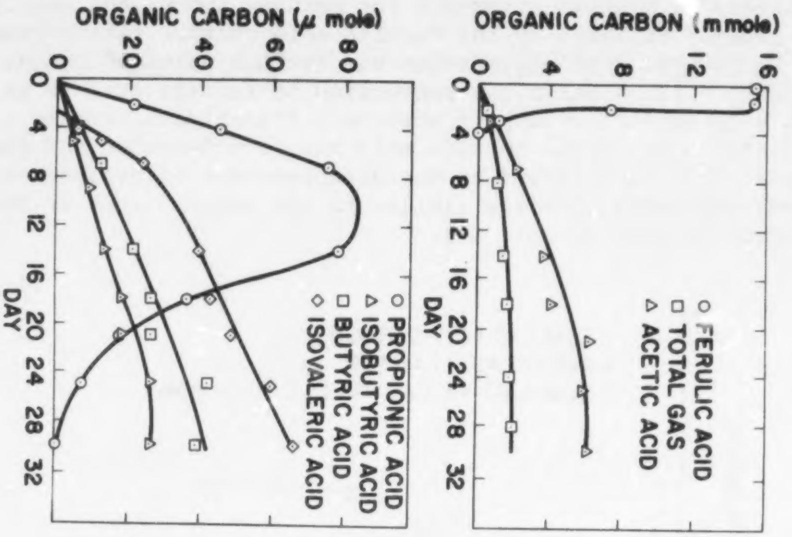

Figure 7. A--Acetate buildup in BESA inhibited ferulic acid cultures.
B--Temporary buildup of propionic acid and appearance of isobutyric, butyric, and isovaleric acids.

DISCUSSION

Methanogenic enrichment cultures are able to degrade a wide range of ligno-aromatic compounds with conversion of the substrate carbon to CO_2 and CH_4. Well acclimated cultures can readily produce stoichiometric amounts of total gas and CH_4 from various aromatic compounds provided as the sole carbon source. The fact that these microbial populations are simultaneously acclimated to more than one aromatic compound suggests that compounds of similar structure may be degraded to methane via similar pathways or partial pathways. In addition, some simultaneously utilized compounds may serve as intermediates during degradation, such as with vanillin or vanillic acid during ferulic acid decomposition.

For methane formation, aliphatic acids and acetate are important precursors and result from cleavage of the ring structure. The role of acetate as a methane precursor is common in other methanogenic systems (12). The immediate uptake of acetate by the ferulic acid culture indicates that an active group of acetate-utilizing methanogens is present.

Methane bacteria are known to utilize a very limited number of simple compounds such as acetate, formate and the reduction of CO_2 to CH_4. The conversion of ligno-aromatic compounds such as ferulic acid, therefore, must rely first on other anaerobic species for initial decomposition, ring cleavage, and formation of intermediate volatile acids. Without these reactions and the production of precursors, methane formation could not take place. Therefore, fermentation of ligno-aromatic compounds to CO_2 and CH_4 is dependent upon a microbial food chain or microbial consortium as termed by Ferry and Wolfe (9).

Propionate, isobutyrate, butyrate, and isovalerate were detected in cultures inhibited with 10^{-4}M BESA, without which detectable levels would not have been obtained. The buildup of propionate (Fig. 7B), followed by its disappearance when acetate buildup leveled off, supports previous reports that propionate is readily converted to acetate and CO_2 (13) and that it is an intermediate in aromatic degradation (2). Since rapid disappearance of ferulic acid was unaffected by the BESA and decomposition of propionate also proceeds, it appears that the BESA has little or no effect on the other organisms in the microbial food chain.

The fate of the ferulic acid molecule may take one or several pathways which is unclear at this point. The presence of isobutyric and isovaleric acid suggests that the pathway(s) may be somewhat different from that which has been elucidated for benzoate (8). An example of the stoichiometry describing conversion of ferulic acid through one of the volatile acid intermediates, isovaleric acid, is shown in Figure 8. The presence of all of the intermediates, except for methanol, has been detected in our enrichment systems. In addition, carbon balance data (Fig. 2) agree closely with the net reaction.

$$\underset{\text{FERULIC ACID}}{C_{10}H_{10}O_4} + 3H_2O + 6H \longrightarrow \underset{\text{ISO-VALERATE}}{C_5H_{10}O_2} + \underset{\text{ACETATE}}{2C_2H_4O_2} + \underset{\text{METHANOL}}{CH_4O}$$

$$\underset{\text{ISO-VALERATE}}{C_5H_{10}O_2} + 4H_2O \longrightarrow 2CH_4 + 3CO_2 + 10H$$

$$2C_2H_4O_2 \longrightarrow 2CH_4 + 2CO_2$$

$$\underset{\text{METHANOL}}{CH_4O} + 2H \longrightarrow CH_4 + H_2O$$

$$\tfrac{1}{4}CO_2 + 2H \longrightarrow \tfrac{1}{4}CH_4 + \tfrac{1}{2}H_2O$$

$$\text{NET: } C_{10}H_{10}O_4 + 5.5H_2O \longrightarrow 4.75CO_2 + 5.25CH_4$$

Figure 8. Stoichiometry of ferulic conversion to CO_2 and CH_4 through isovalerate.

In conclusion, decomposition of ferulic acid to CO_2 and CH_4 appears to be mediated by a consortium of anaerobic organisms. Compounds resulting from ring cleavage include propionate, butyrate, isobutyrate and isovalerate which are likely to be converted to acetate and CO_2, which in turn are used by methanogens for production of carbon dioxide and methane.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Alexander, M. 1965. Biodegradation: problems of molecular recalcitrance and microbial fallibility. Adv. Appl. Microbiol. 7:35.

2. Balba, M. T., and W. C. Evans. 1977. The methanogenic fermentation of aromatic substrates. Biochem. Soc. Trans. 5:302-304.

3. Barker, H. A. 1956. Biological formation of methane. *In* Bacterial fermentations. John Wiley and Sons, New York.

4. Clark, F. M., and L. R. Fina. 1952. The anaerobic decomposition of benzoic acid during methane fermentation. Arch. Biochem. 36:26-32.

5. Dagley, S. 1967. The microbial metabolism of phenolics. *In* A. D. Mclaren and G. H. Peterson (eds.), Soil biochemistry. Edward Arnold, London.

6. Dutton, P. L., and W. C. Evans. 1969. The metabolism of aromatic compounds by *Rhodopseudomonas palustris*. Biochem. J. 113:525-536.

7. Evans, W. C. 1963. The microbiological degradation of aromatic compounds. J. Gen. Microbiol. 32:177-184.

8. Evans, W. C. 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature 270:17-22.

9. Ferry, J. G., and R. S. Wolfe. 1976. Anaerobic degradation of benzoate to methane by a microbial consortium. Arch. Microbiol. 107:33-40.

10. Fina, L. R., and A. M. Fiskin. 1960. The anaerobic decomposition of benzoic acid during methane fermentation. II. Fate of carbons one and seven. Arch. Biochem. Biophys. 91:163-165.

11. Guyer, M., and G. Hegeman. 1969. Evidence for a reductive pathway for the anaerobic metabolism of benzoate. J. Bacteriol. 99:906-907.

12. Jeris, J. S., and P. L. McCarty. 1965. The biochemistry of methane fermentation using C^{14} tracers. J. Water Poll. Contr. Fed. 37:178-192.

13. McCarty, P. L., J. S. Jeris, and W. Murdoch. 1963. Individual volatile acids in anaerobic treatment. J. Water Poll. Contr. Fed. 35:1501-1516.

14. Ornston, L. N., and R. Y. Stanier. 1964. Mechanism of β-keto-adipate formation by bacteria. Nature 204:1279-1283.

15. Pearl, I. A. 1967. The chemistry of lignin. Marcel Dekker, Inc., New York.

16. Proctor, M. H., and S. Scher. 1960. Decomposition of benzoate by a photosynthetic bacterium. Biochem. J. 76:33 pp.

17. Tarvin, D., and A. M. Buswell. 1934. The methane fermentation of organic acids and carbohydrates. J. Am. Chem. Soc. 56:1751-1755.

18. Taylor, B. F., W. L. Campbell, and I. Chinoy. 1970. Anaerobic degradation of the benzene nucleus by a facultatively anaerobic microorganism. J. Bacteriol. 102:430-437.

19. Williams, R. J., and W. C. Evans. 1975. The metabolism of benzoate by *Moraxella* species through anaerobic nitrate respirations: Evidence for a reductive pathway. Biochem. J. 148:1-10.

20. Wolin, E. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. 238:2882-2886.

SOME APPROACHES TO STUDIES ON THE DEGRADATION OF AROMATIC HYDROCARBONS BY FUNGI

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ABSTRACT

A wide taxonomic and phylogenetic spectrum of fungi were shown to transform naphthalene. The ability to oxidize naphthalene predominated in the Mucorales, but significant hydroxylation also occurred in species of *Neurospora*, *Claviceps* and *Psilocybe*. The predominant metabolite formed was 1-naphthol. Other products identified were 4-hydroxy-1-tetralone, *trans*-1,2-dihydroxy-1,2-dihydronaphthalene, 2-naphthol, 1,2- and 1,4-naphthoquinone. *Cunninghamella elegans* oxidized naphthalene, biphenyl and dibenzofuran by reactions similar to those observed with mammalian enzyme systems.

Aromatic hydrocarbons are ubiquitous in nature. They are formed by medium and high temperature pyrolysis of organic material (2). In marine ecosystems, these compounds have been isolated from ocean water, marine sediments and certain marine organisms. Sources include natural submarine seepage of petroleum and accidental oil spillage which occurs in the production, transport, and utilization of petroleum. In addition, trace amounts of polycyclic aromatic hydrocarbons are released into the environment by a wide variety of natural and man-made sources, such as forest fires, incomplete combustion of organic material, motor vehicle emissions, coal-liquefaction and gasification processes, cigarette smoke, and industrial wastes (2).

Studies in our laboratory have been directed toward elucidating mechanisms used by microorganisms for degradation of aromatic hydrocarbons and related compounds. Such investigations have shown that bacteria incorporate one molecule of atmospheric oxygen into the benzenoid nucleus to form dihydrodiols with a relative *cis*-stereochemistry (16, 18). Further oxidation of these intermediates leads to the formation of catechols which are substrates for enzymatic fission of the aromatic ring (12, 29). The above observations are in direct contrast to the mechanisms used by mammalian systems for the oxidation of aromatic hydrocarbons. Mammalian enzymes incorporate one atom of molecular oxygen into the aromatic substrate to form reactive arene oxides which can isomerize to phenols or undergo enzymatic hydration to yield *trans*-dihydrodiols (23, 25, 27). There is ample evidence in the literature to suggest that fungi oxidize aromatic hydrocarbons in a manner similar to that observed in higher organisms (1, 13, 14, 28, 31). Consequently, we have initiated a comprehensive research program to investigate the biodegradative potential of a wide range of fungal species. In addition to providing information on the differences between mammals and microorganisms with respect to oxidation mechanisms, the results are also relevant in terms of the fate and possible effects of aromatic hydrocarbons in the environment. In this communication we summarize some of our results on fungal oxidation of naphthalene and show how the techniques used for the study of this substrate can be extended to other aromatic hydrocarbons.

MATERIALS AND METHODS

ORGANISMS AND GROWTH CONDITIONS

The isolation and characterization of *Cunninghamella elegans* has been described previously (8). The sources of the fungi and the growth conditions used in the survey for naphthalene oxidation also have been reported (11). Small-scale fermentations were conducted in 125-ml Erlenmeyer flasks which contained 30 ml of Sabouraud dextrose broth. After 48 h, cultures were harvested as described previously (9) and transferred to 30 ml of fresh medium. Naphthalene, dibenzofuran and biphenyl were added to separate flasks to give a final concentration of 0.5 mg/ml. Two control flasks were incubated, one with sterile Sabouraud dextrose broth, and the other with sterile dextrose broth and substrate. All flasks were incubated at 30°C for 48 h on a rotary shaker at 250 rpm. At the end of each experiment, cultures were examined microscopically for bacterial contamination. No contamination was observed.

EXTRACTION AND DETECTION OF TRANSFORMATION PRODUCTS

After 48 h the mycelium was filtered, and the culture filtrate extracted with 3 volumes of ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, and the solvent removed in vacuo at 30°C. The residue was examined for metabolic products by thin-layer, high-pressure liquid and silica gel column chromatography and compared with the residues obtained from each control. A schematic representation of the separation methods is depicted in Figure 1.

ANALYTICAL METHODS

All analytical procedures except for those noted below were as previously described (11). High-pressure liquid chromatography (HPLC) was used to separate napththalene, biphenyl and dibenzofuran metabolites. HPLC was performed on a Water Associate Model 6000 A solvent delivery system. A Model U-6K septumless injector and Model 440 absorbance detector operated at 254 nm. Separation was achieved with a μBondapak C_{18} (3.9 mm x 30 cm).

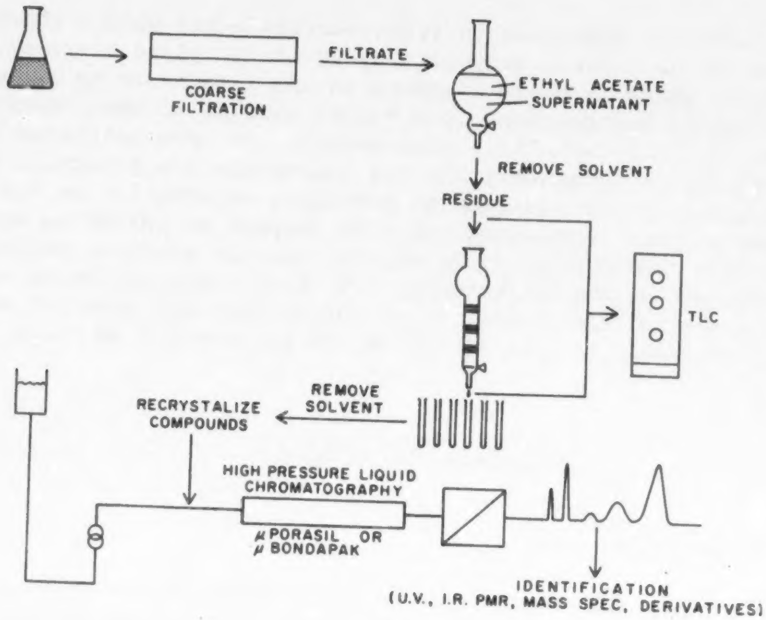

Figure 1. Schematic representation of techniques used to separate, isolate and identify naphthalene, biphenyl and dibenzofuran metabolites.

Naphthalene metabolites were separated by gradient elution. The initial solvent composition was 50% methanol and 50% water. The final solvent concentration was 95% methanol and 5% water. A convex gradient at curvature setting four was employed with a flow rate of 0.4 ml/min. The biphenyl and dibenzofuran metabolites were also separated by gradient elution. The initial solvent composition was 30% acetonitrile and 70% water. The final solvent concentration was 70% acetonitrile and 30% water. A gradiant curvature setting eight was employed at a flow rate of 1.5 ml/min. As metabolites eluted from the column, samples were collected and immediately analyzed by ultraviolet spectrophotometry and mass spectrometry. Extinction coefficients at 254 nm were used to determine the relative amount of each metabolite produced by the different microorganisms.

CHEMICALS

Naphthalene and dibenzofuran were obtained from Aldrich Chemical Company. Biphenyl was obtained from Mallinckrodt Chemical Works. *trans*-1,2-Dihydroxy-1,2-dihydronaphthalene was a generous gift from L. A. Kapicak, Union Carbide. Both *cis*-1,2-dihydroxy-1,2-dihydronaphthalene and 4-hydroxy-1-tetralone were prepared as described previously (19, 9). 1-Naphthol, 2-naphthol, 1,4-naphthoquinone and 1,2-naphthoquinone (J. T. Baker Company) were purified by vacuum sublimation before use. 2-Hydroxydibenzofuran, 3-hydroxydibenzofuran and 4-hydroxydibenzofuran were generous gifts from N. E. Stjernström, Astra Pharmaceuticals AB, Sweden. 2-Hydroxybiphenyl was obtained from Aldrich Chemical Company, and 3-hydroxybiphenyl and 2,5-dihydroxybiphenyl from RFR Corporation. All solvents used for HPLC were purchased from Burdick and Jackson Laboratories, Inc.

RESULTS AND DISCUSSION

Cells of *Cunninghamella elegans* grown in the presence of naphthalene produced six metabolites. The separation of these products by HPLC is shown in Figure 2A. The major metabolites were 1-naphthol (67.9%) and 4-hydroxy-1-tetralone (16.7%). Minor products isolated were 1,4-naphthoquinone (2.8%), 1,2-naphthoquinone (0.2%), 2-naphthol (6.3%), and *trans*-1,2-dihydroxy-1,2-dihydronaphthalene (5.3%). The isolation and identification of the napthalene metabolites has been described earlier (9). It has been well documented that mammals oxidize naphthalene to naphthalene-1,2-oxide, 1-naphthol and *trans*-1, 2-dihydroxy-1,2-dihydronaphthalene (20, 21, 25, 26). Naphthol arises from the intermediate naphthalene-1,2-oxide by spontaneous rearrangement, whereas the *trans*-naphthalene diol arises by enzymatic hydration. In contrast, bacteria metabolize naphthalene to *cis*-dihydrodiols by the action of a dioxygenase (7, 19, 22). Our results suggest that *C. elegans* oxidizes naphthalene in a manner analogous to mammalian enzyme systems. To further demonstrate that the metabolism of naphthalene by *C. elegans* is analogous to mammalian cytochrome P-450 systems, we prepared subcellular fractions of *C. elegans* and assayed for naphthalene oxygenase activity. The metabolites formed from the microsomal hydroxylation of naphthalene were *trans*-1,2-dihydroxy-1,2-dihydronaphthalene, 1-naphthol, and 2-naphthol. The major metabolite was 1-naphthol (10). Enzymatic activity was dependent on the presence of reduced nicotinamide-adenine dinucleotide phosphate and oxygen. Reduced microsomal preparations when treated with carbon monoxide showed absorption maxima at 450 and 420 nm. The results suggest that the metabolism of naphthalene by fungal microsomes may be analogous to the cytochrome P-450-dependent monooxygenase activity associated with mammalian liver microsomes.

The techniques developed during our studies with *C. elegans* permitted a detailed study of naphthalene oxidation in organisms belonging to five major fungal taxa. Eighty-six species of fungi in thirteen classes were investigated. Metabolites were detected by thin layer chromatography and quantitatively analyzed by high pressure liquid chromatography. Approximately 55% of the organisms investigated oxidized naphthalene (Table 1). Naphthalene oxidation predominated in the Zygomycetes. Species of *Cunninghamella, Syncephalastrum* and *Mucor* oxidized naphthalene to similar compounds as described for *C. elegans*. Other fungi showing a similar degradative activity were *Neurospora crassa, Claviceps paspali* and *Psilocybe* strains. Approximately 35% of the fungi tested produced *trans*-naphthalene diol. The fungi which produced significant 1-naphthol levels also showed the production of 4-hydroxy-1-tetralone. This is consistent with our previous study with *C. elegans* (9) which indicated that 4-hydroxy-1-tetralone is formed from 1-naphthol. Previously Bollag et al. (3) had demonstrated the formation of 4-hydroxy-1-tetralone as a bacterial oxidation product of 1-naphthol. *trans*-Naphthalene diol and/or 1-naphthol were produced from some of the lower fungi (Chytridiomycetes and Oomycetes) screened. This suggests that the enzymes responsible for naphthalene metabolism may have originated very early during the evolution of eucaryotic cells. Fungi from a variety of trophic habitats and environments were able to oxidize naphthalene to some extent and in all cases it appears that the reactions are similar to those that occur in mammals.

The techniques described above were also utilized to study the metabolism of biphenyl and dibenzofuran by *C. elegans*.

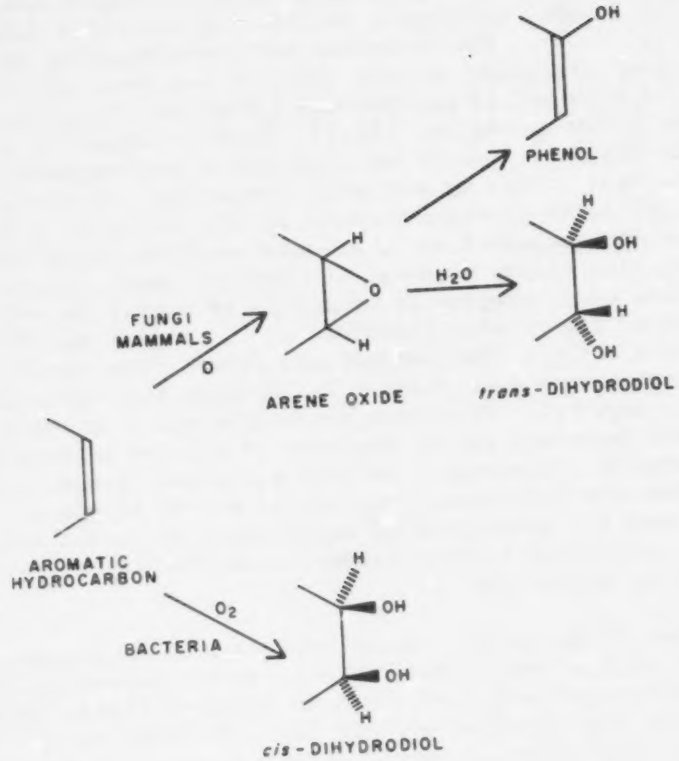

Figure 2. Separation of metabolites formed from naphthalene (A), biphenyl (B) and dibenzofuran (C) by high pressure liquid chromatography. Separation methods described under Materials and Methods.

Biphenyl is used commercially as a fungistat in the shipping of citrus fruits. Although the metabolism of biphenyl by mammals (30) and bacteria (4, 5, 6, 17, 24) has been widely studied, relatively little is known about its metabolism by fungi (28, 31). Biphenyl oxidation by *C. elegans* produced 2-, 3- and 4-hydroxybiphenyl. The major site of hydroxylation is at the 4-position to form 4-hydroxybiphenyl and secondary oxidation also produced significant amounts of 4,4'-dihydroxybiphenyl. Less hydroxylation occurs at the 2- and

3-positions. When 4-hydroxybiphenyl or 2-hydroxybiphenyl replaces biphenyl as the substrate, *C. elegans* produces 4,4'-dihydroxybiphenyl and 2,5-dihydroxybiphenyl, respectively. HPLC analysis of the metabolites produced by *C. elegans* grown in the presence of biphenyl is shown in Figure 2B. In addition to free phenolic metabolites, experiments with ^{14}C-biphenyl indicate that 44% of the known metabolites present in the culture medium are glucuronide conjugates. These results are similar to those formed in the transformation of biphenyl by mammals.

TABLE 1. METABOLITES FORMED FROM NAPHTHALENE BY DIFFERENT SPECIES OF FUNGI

Fungi	Naphthalene metabolites*
Mastigomycota	
Chytridomycetes	
Phlyctochytrium reinboldtae	1,2,5
Rhizophlyctis rosea	5
R. harderi	5
Oomycetes	
Saprolegnia parasitica	5
Phytophthora cinnamomi	5
Thraustochytrium sp.	5
Hyphochytrium catenoides	1,5
Zygomycota	
Zygomycetes	
Cunninghamella elegans	1,2,3,4,5,6
C. echinulata	1,2,3,4,5,6
C. japonica	1,2,3,4,5,6
Syncephalastrum sp.	1,2,3,4,5,6
S. racemosum	1,2,3,4,5,6
Mucor sp.	1,2,3,4,6
M. heimalis	1,2,3,5,6
Gilbertella persicaria	1,5,6
Absidia sp.	1,2,5,6
A. glauca	1,2,5,6
Zygorhynchus moelleri	1,5,6
Cokeromyces poitrassi	1,5,6
Choanephora campincta	1,5,6
Phycomyces blakesleenaus	1,5,6
Circinella sp.	1,5,6
Thamnidium anamolum	1,5,6
Rhizopus arrhizus	1,5,6
Basidiobolus ranarum	1,5,6
Conidiobolus gonimodes	1,5,6
Trichomycetes	
Smittium culisetae	5
S. simulii	5
S. culicis	5
Ascomycota	
Saccharomyces cerevisiae	1,5,6
Emericellopsis sp.	5
Neurospora crassa	1,2,3,4,5,6
Sordaria fimicola	5,6
Claviceps paspali	1,2,3,4,5,6
Basidiomycota	
Psilocybe strictipes	1,2,3,4,5,6
P. subaeruginascens	1,2,3,4,5,6
P. cubensis	1,2,3,4,5,6
P. stuntzii	1,2,3,4,5,6
Panaeolus subbalteatus	5,6
P. cambodginensis	5,6
Deuteromycota	
Aspergillus niger	5,6
Penicillium notatum	5,6
Gliocladium sp.	5,6
Epicoccum nigrium	5,6
Pestalotia sp.	5

*1 = *trans*-naphthalene diol; 2 = 4-hydroxy-1-tetralone; 3 = 1,2-naphthoquinone; 4 = 1,4-naphthoquinone; 5 = 1-naphthol; 6 = 2-naphthol.
Metabolites were separated and quantitated as described previously (11).

Dibenzofuran is an oxygen-containing heteroaromatic compound identified as a constituent of coal tar. Chlorinated derivatives of dibenzofuran have been identified as trace contaminants in commercial preparations of polychlorinated biphenyl, pentachlorophenol and hexachlorobenzene. These chlorinated dibenzofurans have been reported to be more toxic to mammals than the compounds that they contaminate (15). Despite their widespread occurrence and toxicological properties, little is known about the microbial degradation of dibenzofuran. *C. elegans* grown in the presence of dibenzofuran produced four metabolites. The separation of these metabolites by HPLC is shown in Figure 2C. *C. elegans* oxidized dibenzofuran to form *trans*-2,3-dihydroxy-2,3-dihydrodibenzofuran, 2-hydroxydibenzofuran, 3-hydroxydibenzofuran and 2,3-dihydroxydibenzofuran. Each product was isolated and identified by conventional chemical techniques. The formation of 2-hydroxydibenzofuran and 3-hydroxydibenzofuran also suggests the formation of 2,3-dibenzofuran oxide since it is characteristic of arene oxides to spontaneously isomerize to form phenols. These phenols did not arise from the decomposition of *trans*-2,3-dihydroxy-2,3-dihydrodibenzoburan since control experiments indicated that this dihydrodiol was very stable under our culture conditions and isolation techniques.

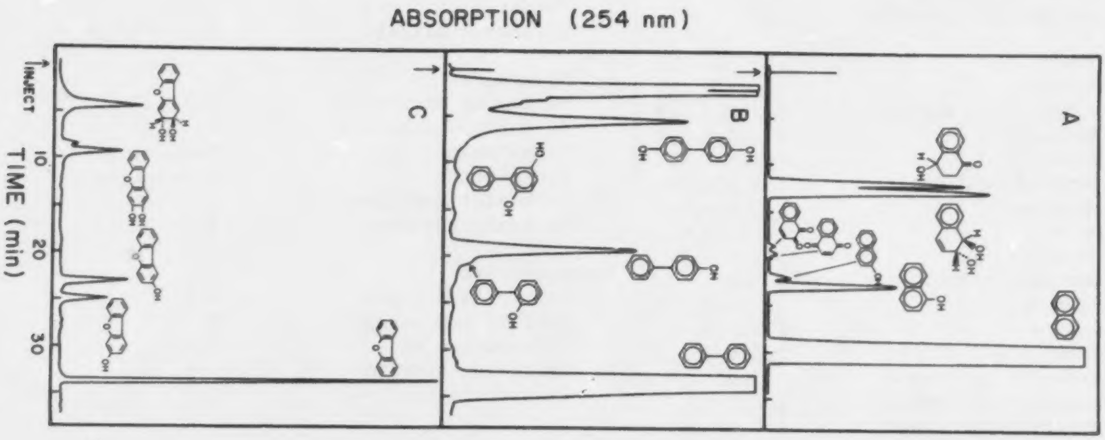

Figure 3. Initial reactions used by mammals, fungi and bacteria to oxidize aromatic hydrocarbons.

This paper provides evidence for the fungal metabolism of aromatic hydrocarbons. The fungi surveyed in this report include organisms found in terrestrial, freshwater and marine ecosystems. Many of the fungi tested oxidized naphthalene to some extent. *C. elegans* oxidized naphthalene, biphenyl and dibenzofuran to metabolites which are quite similar to those reported in mammalian enzyme systems. These mechanisms are compared to the metabolism of aromatic hydrocarbons by bacteria in Figure 3.

The results obtained using pure cultures of fungi, chemically defined media, and simple aromatic substrates, reveal general features of degradation and information that may be valuable in predicting the fate of these and more complex molecules in the environment.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Auret, B. J., D. R. Boyd, P. M. Robinson, C. G. Watson, J. W. Daly, and D. M. Jerina. 1971. The NIH Shift during the hydroxylation of aromatic substrates by fungi. Chem. Commun., p. 1585.

2. Blumer, M. 1976. Polycyclic aromatic hydrocarbons in nature. Sci. Am. 234:34-44.

3. Bollag, J.-M., E. J. Czaplicki, and R. D. Minard. 1975. Bacterial metabolism of 1-naphthol. Agric. Food Chem. 23:85-90.

4. Catelani, D., C. Sorlini, and V. Treccani. 1971. Experientia 27(10): 1173-1174.

5. Catelani, D., A. Colombi, C. Sorlini, and V. Treccani. 1973. Biochem. J. 134:1063-1966.

6. Catelani, D., and A. Colombi. 1974. Metabolism of biphenyl. Biochem. J. 143:431-434.

7. Catterall, F. A., K. Murray, and P. A. Williams. 1971. The configuration of the 1,2-dihydroxy-1,2-dihydronaphthalene formed in the bacterial metabolism of naphthalene. Biochem. Biophys. Acta 237:361-364.

8. Cerniglia, C. E., and J. J. Perry. 1973. Crude oil degradation by microorganisms isolated from the marine environment. Z. Allg. Mikrobiol. 13: 299-306.

9. Cerniglia, C. E., and D. T. Gibson. 1977. Metabolism of naphthalene by *Cunninghamella elegans*. Appl. Envir. Microbiol. 34:363-370.

10. Cerniglia, C. E., and D. T. Gibson. 1978. Metabolism of naphthalene by cell extracts of *Cunninghamella elegans*. Arch. Biochem. Biophys. 186: 121-127.

11. Cerniglia, C. E., R. L. Hebert, P. J. Szaniszlo, and D. T. Gibson. 1978. Fungal transformation of naphthalene. Arch. Microbiol. (in press).

12. Dagley, S. 1971. Catabolism of aromatic compounds by microorganisms. Adv. Microbial Physiol. 6:1-46.

13. Ferris, J. P., M. J. Fasco, F. L. Stylianopoulou, D. M. Jerina, J. W. Daly, and A. M. Jeffrey. 1973. Monooxygenase activity in *Cunninghamella bainieri*: Evidence for a fungal system similar to liver microcosms. Arch. Biochem. Biophys. 156-97-103.

14. Ferris, J. P., L. H. MacDonald, M. A. Patrie, and M. A. Martin. 1976. Aryl hydrocarbon hydroxylase activity in the fungus *Cunninghamella bainieri*: Evidence for the presence of cytochrome P-450. Arch. Biochem. Biophys. 174:443-452.

15. Fishbein, L. 1976. Trace organic contaminants in the environment. I. Chlorinated dioxins and dibenzofurans. Int. J. Ecol. Environ. Sci. 2: 69-81.

16. Gibson, D. T. 1972. The microbial oxidation of aromatic hydrocarbons. Crit. Rev. Microbiol. 1:199-223.

17. Gibson, D. T., R. L. Roberts, M. C. Wells, and V. M. Kobal. 1973. Oxidation of biphenyl by a *Beijerinckia* species. Biochem. Biophys. Res. Comm. 50:211-219.

18. Gibson, D. T. 1977. Biodegradation of aromatic petroleum hydrocarbons p. 36-46. *In* D. A. Wolfe, ed.), Fate and effects of petroleum hydrocarbons. Pergamon Press, New York.

19. Jeffrey, A. M., H. J. C. Yeh, D. M. Jerina, T. R. Patel, J. F. Davey, and D. T. Gibson. 1975. Initial reactions in the oxidation of naphthalene by *Pseudomonas putida*. Biochemistry 14:575-584.

20. Jerina, D. M., J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg, and S. Udenfriend. 1968. The role of oxide-oxepin systems in the metabolism of aromatic substrates. III. Formation of 1,2-naphthalene oxide from naphthalene by liver microsomes. J. Am. Chem. Soc. 90:6525-6527.

21. Jerina, D. M., J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg, and S. Udenfriend. 1970. 1,2-naphthalene oxide as an intermediate in the microsomal hydroxylation of naphthalene. Biochemistry 9:147-156.

22. Jerina, D. M., J. W. Daly, A. M. Jeffrey, and D. T. Gibson. 1971. *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene: a bacterial metabolite from naphthalene. Arch. Biochem. Biophys. 142:394-396.

23. Jerina, D. M., and J. W. Daly. 1974. Arene oxides: a new aspect of drug metabolism. Science 185:573-582.

24. Lunt, D., and W. C. Evans. 1970. The microbial metabolism of biphenyl. Biochem. J. 118:54p-55p.

25. Oesch, F., D. M. Jerina, and J. W. Daly. 1971. Conversion of naphthalene to *trans*-naphthalene dihydrodiol: Evidence for the presence of a coupled aryl monooxygenase-epoxide hydrase system in hepatic microsomes. Biochem. Biophys. Res. Comm. 46:1713-1720.

26. Oesch, F., D. M. Jerina, J. W. Daly, A. Y. H. Lu, R. Kuntzman, and A. H. Conney. 1972. A reconstituted microsomal enzyme system that converts naphthalene to *trans*-1,2-dihydroxy-1,2-dihydronaphthalene via napththalene-1,2-oxide: Presence of epoxide hydrase in cytochrome P-450 and P-448 fractions. Arch. Biochem. Biophys. 153:62-67.

27. Oesch, F. 1973. Mammalian epoxide hydrases: Inducible enzymes catalyzing the inactivation of carcinogenic metabolites derived from aromatic and olefin compounds. Xenobiotica 3:305-340.

28. Smith, R. V., and J. P. Rosazza. 1974. Microbial models of mammalian metabolism. Aromatic hydroxylation. Arch. Biochem. Biophys. 161: 551-558.

29. Stanier, R. Y., and L. N. Ornston. 1974. The β-ketoadipate pathway. Adv. Microbial Physiol. 9:89-151.

30. Sundström, G., O. Hutzinger, and S. Safe. 1976. The metabolism of chlorobiphenyls--a review. Chemosphere 5:267-298.

31. Wiseman, A., J. A. Gondal, and P. Sims. 1975. 4'-Hydroxylation of biphenyl by yeast containing cytochrome P-450: Radiation and thermal stability, comparisons with liver enzyme (oxidized and reduced forms). Biochem. Soc. Trans. 3:278-281.

A NOVEL SELECTIVE ENRICHMENT TECHNIQUE FOR USE IN BIODEGRADATION STUDIES

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ABSTRACT

A selective enrichment technique has been developed for rapid isolation of lipophilic compound-degrading microorganisms. This method is based on the fact that biodegradation of lipophilic substances takes place mainly at the substance-water interface. Thus, the areal extent of this interface will determine the availability of substrate to the degrading microorganisms and thereby control the primary biodegradation rate as well as the microbial biomass. The isolation procedure involves finely emulsifying the test substance and stabilization of the emulsion with sodium ligninsulfonate. Microorganisms capable of degrading *n*-alkanes, aromatic hydrocarbons, phenols and polychlorinated biphenyls were isolated using this technique.

INTRODUCTION

By definition, an enrichment technique implies the creation of an artificial environment which will either permit the desirable microorganisms to multiply at a faster rate than others or will suppress the growth of all but the desirable microorganisms (1). Enrichment techniques have made an important contribution to development of modern microbiology in studies of microbial degradation and pathways. The literature on enrichment technique is vast and widely scattered, however, most procedures deal with isolation of microorganisms which will grow on hydrophilic compounds. Only limited information is available on isolation of microorganisms capable of degrading lipophilic compounds.

As more and more new organic compounds are being synthesized, the threat of environmental pollution caused by these new chemicals increases accordingly. Most are of lipophilic nature (hydrophobic) which are inherently more resistant to biodegradation than are hydrophilic ones. This is probably due to the requirement of most microorganisms for an aqueous phase to carry out their metabolic activity; furthermore, the hydrophobic nature of these new chemicals makes them less susceptible to microbial attack. Studies in our laboratories have been concerned with the biodegradation of persistent toxic organic substances and the isolation of lipophilic-compound-degrading microorganisms by an enrichment technique.

MATERIALS AND METHODS

Source of Inoculum.--Activated sludge from the local municipal sewage plant was used as the major source of inoculum in the biodegrdation study since the bacterial types (*Alcaligenes*, *Acinetobacter*, *Flavobacterium*, *Pseudomonas* and *Escherichia*) and their concentration (7×10^7 to 2×10^8 ml^{-1}) in the sludge were observed to remain relatively stable throughout the year. Occasionally, samples of activated sludge or soil from local industrial plants also were used as the bacterial seedings.

Growth Medium.--To control the variables normally encountered in a growth medium containing complex organic nutrients such as yeast extract and peptone, the following chemically defined mineral broth was chosen as the basal medium (g $liter^{-1}$): K_2HPO_4, 1.3; KH_2PO_4, 0.82; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.01; $CoCl_2 \cdot 6H_2O$, 0.001; $MnCl_2 \cdot 4H_2O$, 0.001; NaCl, 0.05. The final pH was 6.9. The medium was sterilized at 121°C for 15 min. Sodium ligninsulfonate and the test substances were sterilized separately before being added to the basal medium.

Preparation of Emulsion.--One hundred milligrams of the test substance was added to a sonic cup containing 10 ml of distilled water and 5 mg of sodium ligninsulfonate. Solid test substance was dissolved in a minimal amount of *n*-hexane. The mixture was subjected to pulse ultra-sonification for approximately 1 min until it was emulsified. The emulsion was freshly prepared prior to the initiation of each experiment.

Preparation of Emulsion-Agar Plate.--Fifty milligrams of the test substance were made into 10 ml of a fine emulsion as described above under aseptic conditions, and the emulsion was then mixed with 500 ml of sterile basal agar (approx. 50°C) for pour plate. Plates made in this manner were semi-translucent and colony development was slow (2-7 d).

Enrichment Culture.--Various amounts of the test substance in emulsion form were added to 125-ml Erlenmeyer flasks containing 50 ml of mineral medium and 5 mg of sodium ligninsulfonate to give final concentrations of the test substance of 10, 20, 50, 100 and 300 mg $liter^{-1}$. Fresh activated sludge (0.1 ml) was added to each flask as the inoculum, with incubation at room temperature (21°C) on a gyratory shaker for 1-4 weeks or longer. The samples were checked daily under a phase contrast microscope and the test substance was monitored for biodegradation using gas chromatography. If evidence of biodegradation was noticed, the culture broth was then used for isolation of the degrading microorganism on emulsion agar plates.

Biodegradation Measurement.--In all experiments, only the primary biodegradation of the test substance was followed, i.e., the loss of parent compounds. Five-milliliter samples of the culture broth and 1 ml of *n*-hexane were mixed vigorously in a 15-ml conical glass centrifuge tube on a vortex mixer for 1 min. The emulsion was broken by centrifugation at 2,000 x g for 10 min and the clear hexane extract was used for GC analysis, using two gas chromatographs equipped with FID detectors. A Beckman GC-65 was fitted with dual 1.8 m x 6.3 mm o.d. stainless steel columns containing 2% OV-1 on Chromsorb G-AW-DMCH. A Hewlett-Packard 5730A was fitted with dual 1.8 m x 6.3 mm o.d. glass columns containing 10% OV-1 on Chromsorb W-HP. The oven temperature was programmed from 100-250°C at 5-8°C min^{-1} and the temperatures at the injector port and detector were at 250 and 300°C, respectively.

Growth Determination.--Cultures were in 2-liter Bellco spinner flasks at room temperature (21°C) and at various intervals samples were withdrawn for dry weight determination (5).

Manometric Technique.--Oxygen consumption was measured at 20°C in a Gilson differential respirometer. Cells were harvested from the growth culture, washed and resuspended in cold 0.05 M phosphate buffer (pH 7.0). A typical reaction mixture contained 1 ml of cells, 1 ml of 0.05 M phosphate buffer, 0.9 ml of water, 0.1 ml of substrate and 0.15 ml of 20% KOH in the center well for CO_2 absorption. The final fluid volume was 3.2 ml.

RESULTS

The first successful application of this enrichment technique in this laboratory involved isolation of a PCB-degrading *Pseudomonas* sp. from the activated sludge. The development of the technique for rapid isolation of lipophilic compound-degrading microorganisms was explored by using this bacterium and the commercial PCB mixture (Aroclor 1221). Initially, an attempt was made to incorporate the PCB into the growth medium by dissolving it in an appropriate solvent such as acetone. As expected, the major portion of the added PCB separated out as soon as the acetone solution was added. No significant degradation of PCB was noticed in the growth medium in spite of microbial growth. Further investigation revealed that most sewage microorganisms could utilize acetone as a source of carbon and energy for growth. Microorganisms in activated sludge were capable of rapidly oxidizing acetone or acetate (Fig. 1), thus, it is inadvisable to use acetone as solvent in the enrichment technique since it may serve as an alternate carbon source.

When ultrasonically treated PCB (as emulsion) was added to the mineral medium containing sodium ligninsulfonate as an emulsion stabilizer, significant degradation of PCB (34%) was detected within 9 d. Further incubation to 11 and 14 d resulted in 71% and 99% biodegradation of the PCB, respectively (Fig. 2). From this culture, a PCB-degrading *Pseudomonas* sp. 7509 capable of degrading PCB from concentration of 300 mg $liter^{-1}$ to a level of less than 1 mg $liter^{-1}$ within 48 h was isolated. However, the growth rate of the pseudomonad was greatly retarded if non-emulsified PCB was employed in the growth medium. Examination by phase contrast microscopy of cultures grown with unemulsified PCB revealed that the cells were intimately associated with the dispersion of the PCB droplets in the medium (Fig. 3). Most of the PCB droplets were surrounded by the bacterial cells indicating the possible involvement of bacteria in dispersion of PCB droplets in the growth medium. Examination with interference phase contrast microscopy showed that the cells actually attached themselves to the surface of PCB droplets, suggesting that microbial degradation of PCB could be occurring at the PCB-water interface (Fig. 4). Similar observations were also noticed when this enrichment technique was applied to isolation of other lipophilic compound-degrading microorganisms such as a *p*-cresol-degrading bacterium (Fig. 5). In a medium with PCB as the only carbon and energy source, pseudomonad 7509 gave a near-linear cell yield as a function of concentration of PCB emulsion (Fig. 6).

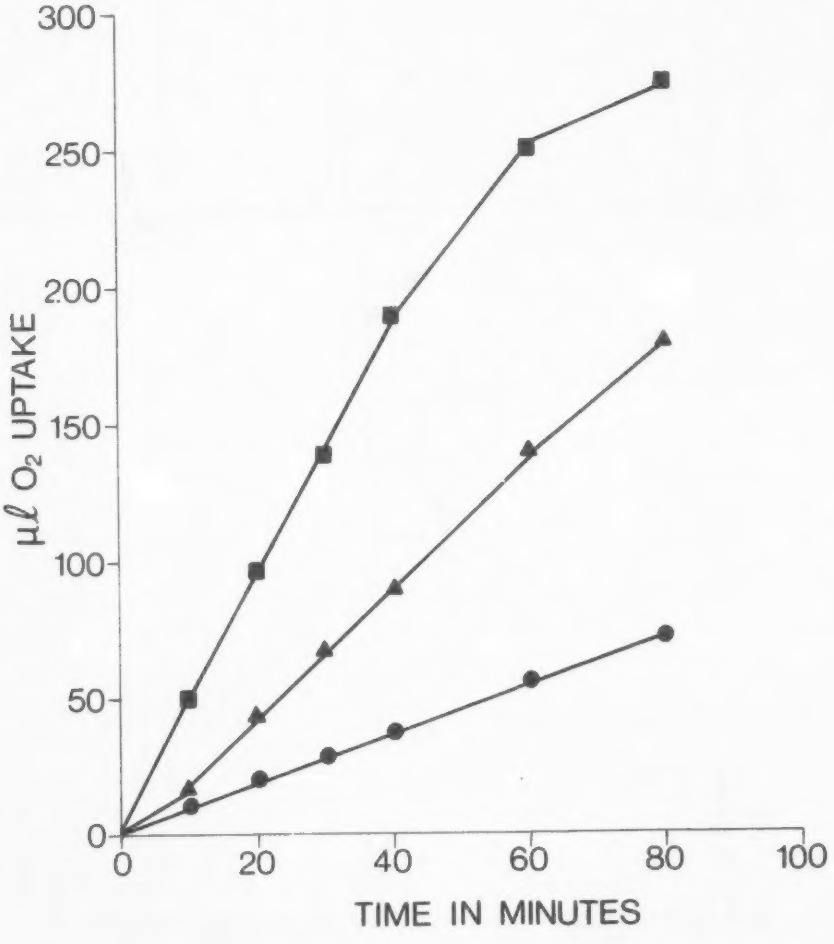


Figure 1. Oxidation of acetate and acetone by activated sludge.
—●— Endogenous; —■— Acetate; —▲— Acetone

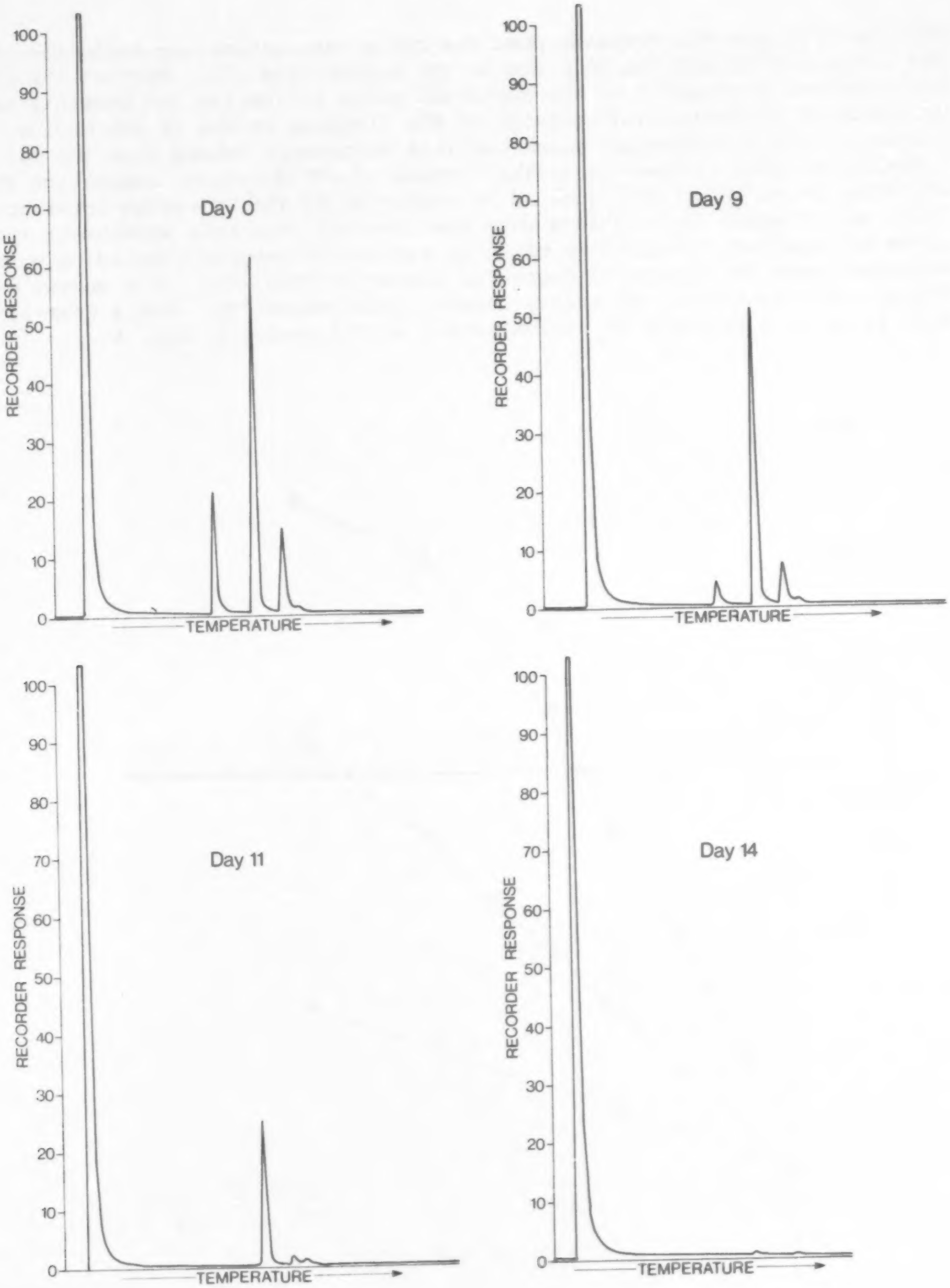
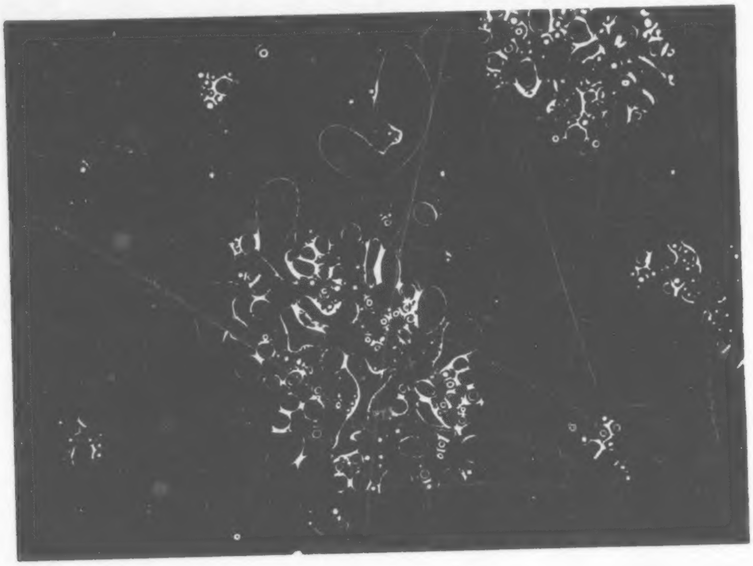
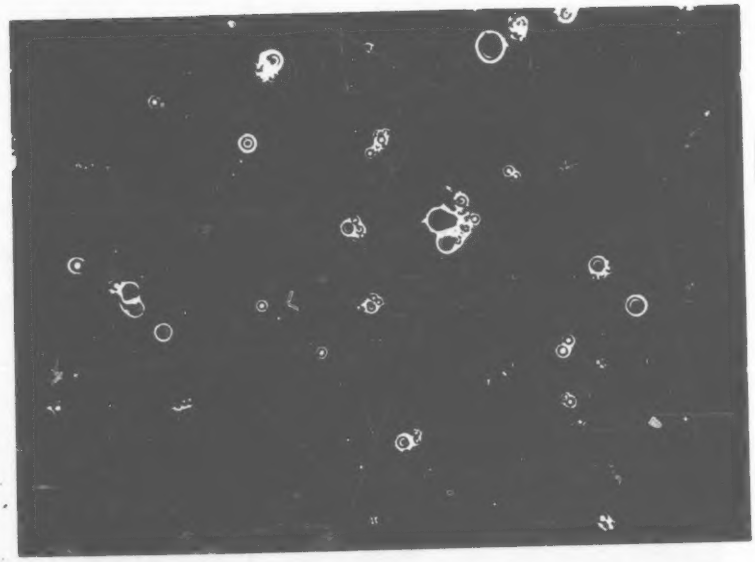


Figure 2. FID gas chromatograms of *n*-hexane extracts of Aroclor 1221 at zero time, after 9 days, 11 days and 14 days incubation with activated sludge.



A



B

Figure 3. Micrographs of the culture broth taken at different growth stages showing the dissociation of PCB droplets. A = early log phase; B = middle log phase. Phase contrast at 400X.

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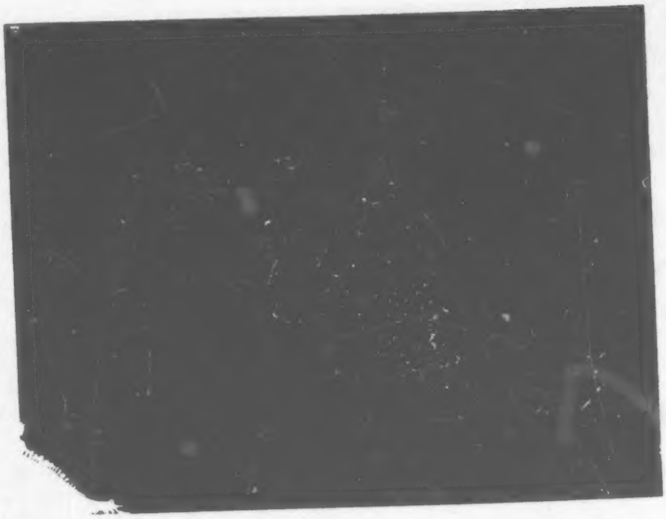

Figure 4. Interference phase micrograph showing the bacterial growth at the PCB-water interface. Interference phase contrast at 1000X.

Figure 5. Interference phase micrograph showing the growth of a *p*-cresol degrading bacterium at the *p*-cresol-water interface (1000X).

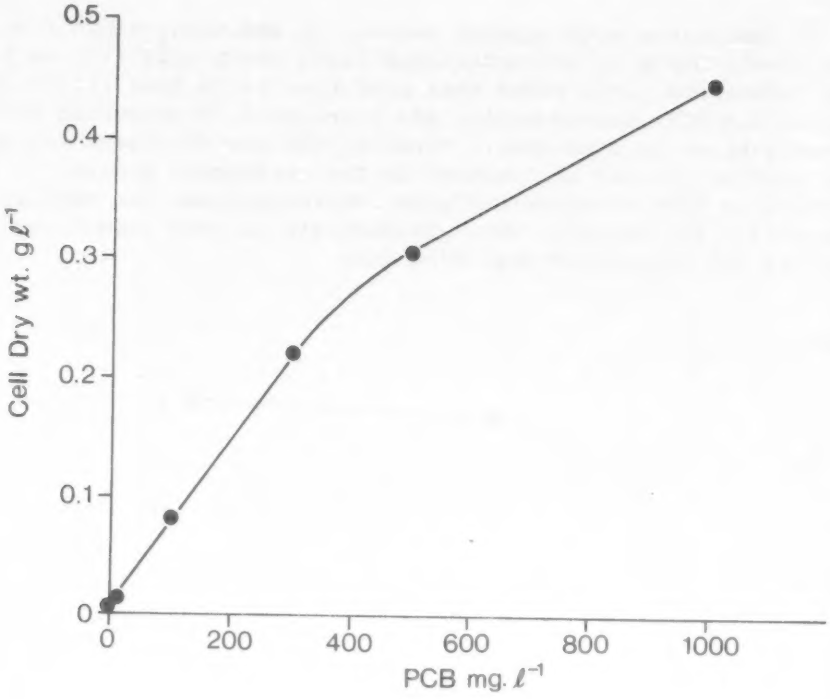

Figure 6. The growth yield of *Pseudomonas* sp. 7509 as a function of Aroclor 1221 concentration in the growth medium.

The rate of PCB degradation by bacteria was also followed by the rate of oxygen consumption (2). The relationship between oxygen consumption and the concentration of PCB in the flask, i.e., the amount of oxygen used by the cells, was a direct function of the concentration of PCB (Fig. 7). For all PCB concentrations employed, there was no lag period for oxygen utilization, suggesting that the bacterial cells oxidized PCB immediately, i.e., the cells grown by this enrichment method possessed vitality sufficient to attack the lipophilic compound. The UV-VIS spectrum of sodium ligninsulfonate recovered from the culture broth showed little difference from the control, indicating that sodium ligninsulfonate was not degraded in the process and hence did not provide an alternate carbon and energy source for the cells.

DISCUSSION

Investigators studying biodegradation of lipophilic substances in the aquatic environment have been confronted with the problem of finding a means to dissolve or disperse such substances in growth media. A number of enrichment methods have been used for isolation of PCB-degrading bacteria, including dissolving of PCB in acetone (6, 8), ethanol (7) and diethyl ether (3). All of these methods have their drawbacks. Acetone and ethanol are good substrates for microorganisms and provide alternate carbon sources for bacterial growth.

Diethyl ether is immiscible with aqueous media. In addition, since PCB cannot be kept in the growth media at concentrations above their solubilities by the aforementioned techniques, this means that bacterial cells have little opportunity to contact the PCB; consequently, the likelihood of enhancing PCB-degrading microorganisms is decreased. Finally, the use of organic carbon-rich nutrients such as glucose and peptone in the enrichment medium (7) is of questionable value in that numerous different microorganisms can readily utilize such nutrients. The bacteria which predominate in such growth media need not necessarily be the lipophilic degrading ones.

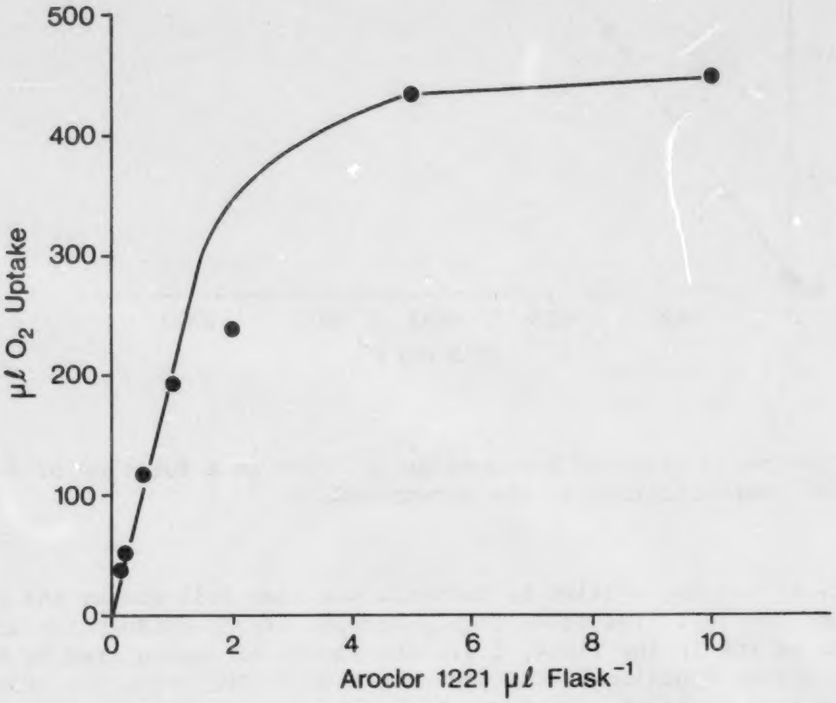

Figure 7. Oxygen uptake as a function of the Aroclor 1221 concentrations in the flask.

The enrichment technique described here avoids most of the problems encountered with previous methods. The production of a fine emulsion of the lipophilic substance through ultrasonic means provides a much larger lipophile water interfacial area, thus allowing the microorganisms to overcome the substrate-limiting aspect which may determine subsequent bacterial growth rate. Sodium ligninsulfonate is used rather than commercial emulsifiers due to its recalcitrance to microbial action. Therefore, most microorganisms isolated by this technique are very active in degradation of the lipophilic compounds.

However, this enrichment technique is useful only when the test substance can be metabolized by the microorganism in question. Certain lipophilic substances, such as DDT, are only degraded by cometabolism (4). However, if necessary, a cometabolite can be applied in the presently described enrichment technique. The latter can be used reliably to obtain some lipophilic substance-degrading microorganisms from the environment.

LITERATURE CITED

1. Aaronson, S. 1970. Experimental microbial ecology. Academic Press, New York.

2. Ahmed, M., and D. D. Focht. 1973. Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. 19:47-52.

3. Baxter, R. A., P. E. Gilbert, R. A. Lidgett, J. H. Mainprize, and H. A. Vodden. 1975. The degradation of polychlorinated biphenyls by microorganisms. Sci. Total Environ. 4:53-61.

4. Focht, D. D., and M. Alexander. 1971. Aerobic cometabolism of DDT analogues by *Hydrogenomonas* sp. J. Agric. Food Chem. 19:20-22.

5. Liu, D. 1973. Microbial degradation of crude oil and the various hydrocarbon derivatives, p. 95-104. *In* D. G. Ahearn and S. P. Meyers (eds.), The microbial degradation of oil pollutants. Center for Wetland Resources, Louisiana State University, Baton Rouge.

6. Sayler, G. S., M. Shon, and R. R. Colwell. 1977. Growth of an estuarine *Pseudomonas* sp. on polychlorinated biphenyl. Microbial Ecol. 3:241-255.

7. Tucker, E. S., V. W. Saeger, and O. Hicks. 1975. Activated sludge primary biodegradation of polychlorinated biphenyls. Bull. Environ. Contamin. Toxicol. 14:705-713.

8. Wong, P. T. S., and K. L. E. Kaiser. 1975. Bacterial degradation of polychlorinated biphenyls. II. Rate studies. Bull. Environ. Contamin. Toxicol. 13:249-256.

DISTRIBUTION, ABUNDANCE, AND PETROLEUM-DEGRADING POTENTIAL OF MARINE BACTERIA FROM MIDDLE ATLANTIC CONTINENTAL SHELF WATERS

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ABSTRACT

Bacterial populations indigenous to surface (1 m) waters of the Middle Atlantic Continental Shelf were sampled at seasonal intervals to determine the abundance and distribution of petroleum-degrading (HC), chitinoclastic, and "total" heterotrophic (HET) bacteria. Simultaneously, unweathered South Louisiana crude oil was added to aliquots of 1-m water samples to evaluate rates and patterns of saturated paraffin degradation on shipboard under controlled incubation conditions. Degradation was examined under selected nutrient and temperature regimes in both closed flasks and prototype "open" or continuous dilution systems. HET bacterial levels generally decreased with distance from land. HC bacteria were most abundant in the coastal boundary layer. Changes in values of HC/HET tended to be directly related to the abundance of petroleum-degrading bacteria. Degradation of saturated paraffins in closed flasks characteristically resulted in removal of *n*-alkanes and isoprenoids at rates related to season, sampling location, and nutrient regime.

Coincident with proposed leasing of tracts for oil exploration on the Middle Atlantic Continental Shelf (MACS), we were given the opportunity to assess the seasonal distribution, abundance and relative in-vitro petroleum-degrading capabilities of marine bacteria. Of particular interest were how viable counts of petroleum-degrading bacteria and the ratio of petroleum-degrading to "total" heterotrophic bacteria varied with season and proximity to land. Previous authors (3, 6, 9, 10, 11, 16, 17) have indicated that in areas of increased levels of pollution by petroleum or related compounds, the viable counts of petroleum-degrading bacteria were elevated. Similarly, increases in the value of the ratio of petroleum-degrading to "total" heterotrophic bacteria have been observed under these conditions (6, 15).

Although viable counts of petroleum-degrading bacteria and/or increase in the ratio of this group to "total" heterotrophs may be indicative of the presence of petroleum (or allied compounds), these values yield no direct information concerning the potential of bacteria to degrade petroleum. Therefore, a sequence of closed flask "batch" degradation experiments were performed to assess the influence of season, inorganic enrichment, and water type on on invitro degradation of South Louisiana crude oil. Finally, to overcome several cultural limitations inherent in closed flask systems, we developed and performed a series of preliminary studies with an "open" or continuous dilution system for simulation of microbial weathering of petroleum at sea.

MATERIALS AND METHODS

SAMPLING

Surface water samples were collected during the four biological seasons at selected stations (Fig. 1) at 1-2 m depth using Niskin sterile bag samplers. Precautions were taken to prevent contamination by discharge from the vessel. Water samples were immediately processed for enumeration of "total" heterotrophic and petroleum-degrading bacteria. Appropriate dilutions or con~~centrations on membrane filters were prepared prior to enumeration.~~

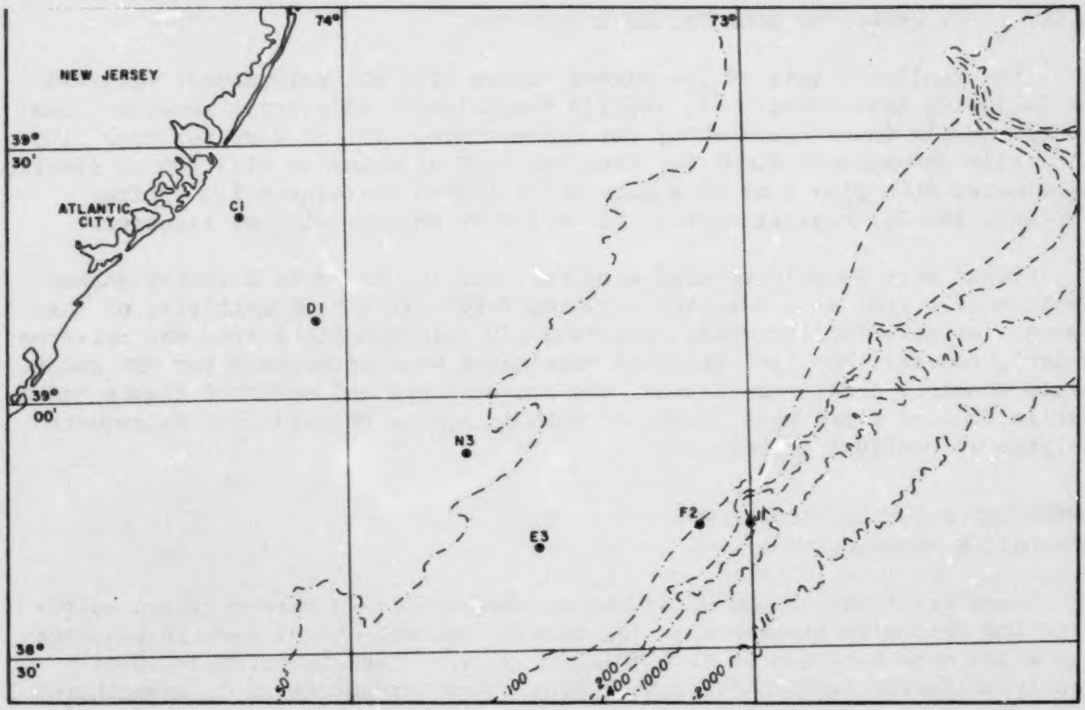

Figure 1. Sampling stations, Middle Atlantic continental shelf waters.

ENUMERATION

Seawater inocula or appropriate dilutions or concentrates thereof were enumerated for "total" heterotrophic bacteria (HET) using a three-tube MPN technique (7) in a heterotrophic medium (HM) modified after ZoBell's 2216E to reduce inorganic precipitate formation. This medium consisted of 1.0 g/liter peptone, 0.5 g/liter yeast extract, 0.01 g/liter ferric citrate, 0.1 g/liter sodium glycerol phosphate, and 1000 ml of aged seawater (final pH of 7.8 after sterilization). Petroleum-degrading bacteria (HC) were also enumerated using a three-tube MPN technique (5) employing a minimal salts enriched seawater (ESWB) containing 1.0 g/liter $(NH_4)_2SO_4$ and 0.1 g/liter K_2HPO_4 in 1000 ml of aged seawater. Following inoculation, approximately 1% sterile unweathered South Louisiana crude oil (SLCO) was added as the carbon source. Petroleum was sterilized by filtration through a 0.4 µ polycarbonate membrane. All media were autoclaved at 121°C for 15 min.

HM tubes were incubated at 20-22°C for two weeks and examined at weekly intervals. ESWB + petroleum (ESWBP) tubes were incubated at similar temperatures on a rotary shaker platform (140 rpm) for one month and examined biweekly. HM tubes were scored positive when turbid; ESWBP tubes were scored positive when turbid or if the oil showed obvious signs of degradation with associated cell debris, or a combination of both. MPN values were calculated using standard tables (1).

CLOSED FLASK PETROLEUM DEGRADATION EXPERIMENTS

Four replicate sets of pre-washed flasks (250 ml, Erlenmeyer) received the following treatments: (a) sterile control--100 ml sterile seawater plus 100 µl sterile unweathered SLCO; (b) non-enriched--100 ml seawater plus 100 µl sterile unweathered SLCO; (c) enriched--100 ml seawater plus 100 µl sterile unweathered SLCO plus 1 ml of a nutrient solution containing 1.0 g/liter $(NH_4)_2SO_4$ and 0.1 g/liter K_2HPO_4; (d) oil-free control--100 ml seawater.

Flasks were loosely covered with foil and incubated on a rotary shaker platform (120 rpm) at a selected seawater temperature representative of that season. At selected intervals, one randomly selected flask from the oil-free control, non-enriched, and enriched treatments were enumerated for HET and HC viable counts. After enumeration, the non-enriched and enriched flasks and a sterile control flask were "pickled" with methylene chloride for subsequent analysis of residual petroleum.

CONTINUOUS DILUTION PETROLEUM DEGRADATION EXPERIMENTS

Three replicate groups of 500 ml Erlenmeyer flasks were arranged to receive the following treatments: (a) sterile control--50 ml sterile seawater plus a low concentration of SLCO (0.01-0.1%, v/v); (b) inoculated--50 ml freshly collected "ambient" seawater plus a low concentration of unweathered SLCO (0.01-0.1%, v/v); (c) oil-free control--50 ml freshly collected "ambient" seawater.

Following the initial inoculation, flasks were incubated on an Orbit

Environ-Shaker 18 (Lab-Line Instruments, Inc.) at 100 rpm and average seasonal ambient temperature. During incubation, flask contents were diluted by adding seawater at the rate of 1 ml/h. Seawater was pumped using a multi-channel peristaltic pump (Desaga, Brinkman Instruments) from two reservoirs. One reservoir contained freshly collected "ambient" seawater and the other contained seawater + 1% formalin. Reservoirs were replenished at regular intervals (Fig. 2). At various incubation intervals, representative flasks for each experimental treatment were randomly harvested and HET and HC enumerated. Glass distilled methylene chloride was then added to the inoculated and sterile control flasks as a "pickling" agent in preparation for analysis of the residual oil.

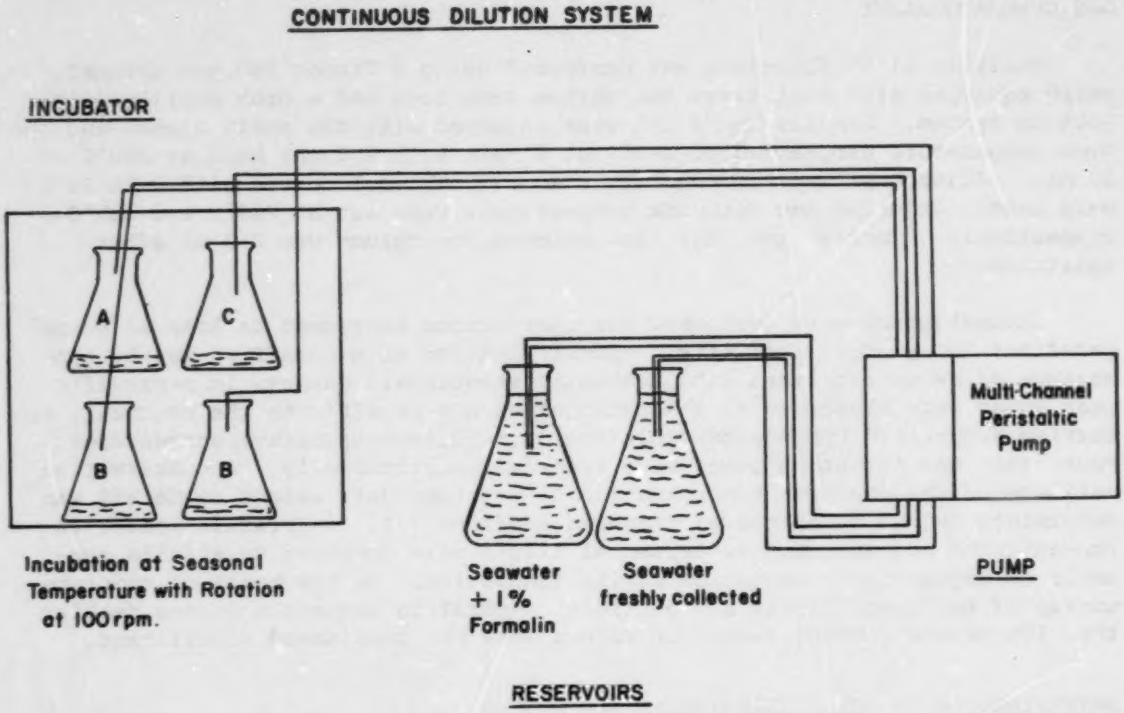
CONTINUOUS DILUTION SYSTEM
INCUBATOR
A
C
B
B
Incubation at Seasonal Temperature with Rotation at 100 rpm.
Seawater + 1% Formalin
Seawater freshly collected
Multi-Channel Peristaltic Pump
PUMP
RESERVOIRS

Figure 2. Schematic of prototype "open" or continuous dilution system.

EXTRACTION OF RESIDUAL PETROLEUM

"Pickled" flasks were acidified (pH 3.0-4.0) with HCl and extracted with two 25-ml portions of methylene chloride. Methylene chloride phases were combined and washed with 50 ml of acidified distilled water (pH 4.0) containing 3% NaCl. After phase separation, the organic phase was dried by passage through anhydrous sodium sulfate and collected in a clean, solvent-washed, pre-weighed evaporation flask. Solvent was then removed by aspiration at 40°C using a McLeod gauge to instantly detect complete solvent removal. The extracted residue was weighed and dissolved in 1 ml of hexane.

FRACTIONATION OF RESIDUES

Extracts were fractionated by column chromatography. Glass columns (1 cm diameter) packed to a height of 17.5 cm with activated silca gel (heated at 235°C for 16 h) were pre-washed with four (4) 10-ml portions of glass distilled hexane. Extracts were then placed on columns in 1 ml hexane and eluted with 17 ml hexane. The first 5 ml of hexane eluting were discarded and the next 13 ml hexane, designated the H2 fraction and containing saturates, were saved. Columns were next eluted with 30 ml of a hexane/benzene (40/60 v/v) mixture and an aromatic-containing fraction collected. Fractions were dissolved in small volumes of hexane and stored at -4°C for subsequent analysis.

GAS CHROMATOGRAPHY

Analysis of H2 fractions was performed using a Tracor 560 gas chromatograph equipped with dual flame ionization detectors and a Grob capillary injection system. Samples (2-2.5 μl) were injected with the split closed and the oven temperature programmed to 240°C at 4°/min with a final hold at 240°C of 10 min. Glass capillary columns (20 m x 0.33 mm i.d.) coated with 0.2% SE-52 were used. Injector and detector temperatures were set at 240°C and 325°C, respectively. Carrier gas (He) flow through the column was 2-3 ml after splitting.

Chromatograms were evaluated for degradation expressed as loss of normal paraffins (nC_{10}-nC_{25}, inclusive). Identification of *n*-paraffins was by comparison of retention times with authentic standards. Changes in *n*-paraffin peak areas were expressed as the ratio of each *n*-paraffin to the naturally occurring isoprenoid hydrocarbon pristane (2,6,10,14-tetramethylpentadecane). Peak areas and retention times were recorded electronically. A conservative estimate of the absolute concentration of pristane/unit weight crude oil was determined by the technique of standard addition (8). *n*-Paraffin losses in non-enriched and enriched experimental flasks were compared to sterile controls to adjust for evaporation during incubation. On the basis of complete workup of replicate blanks and controls, *n*-paraffin summation losses smaller than 10% of the control summation values were not considered significant.

DETERMINATION OF CHEMICAL-PHYSICAL PARAMETERS

ATP concentrations were measured by the method of Strickland and Parsons (12). Concentrations of dissolved organic carbon, nitrates, phosphates, dissolved aliphatic and aromatic hydrocarbons were obtained from relevant chemical and biological benchmark data (14, 15).

RESULTS

Figure 1 is of the (MACS) study area and indicates the locations of 1-m surface water stations. Station C1 was located east of Atlantic City in the coastal boundary layer. Stations D1, N3, and E3 were situated in inner and outer shelf waters. F2 was located in the shelf break region (100-m contour) and J1 was located in slope water. Seasonal variations in water type were noted at station F2 which was frequently located in a frontal zone caused by mixing of shelf and slope water. Water types at Station J1 were sometimes characterized as eddies from the Gulf Stream.

Results from enumeration of viable counts of petroleum-degrading and "total" heterotrophic bacteria are shown in Figures 3 and 4 and Tables 1 and 2. Generally, values of heterotrophs decreased with increased distance from land, with counts falling from about 10^5 bacterial units/ml to 10^2 bacterial units/ml. Geometric means values (Tables 3 and 4) for all transect HET counts did not vary much with season. Considered on a station basis, however, geometric mean values tended to decrease directly with distance from land.

TABLE 1. BACTERIAL COUNTS AND VALUES OF SELECTED CHEMICAL-PHYSICAL PARAMETERS FOR ONE-METER SURFACE WATER SAMPLES ALONG THE SOUTHERN NEW JERSEY TRANSECT DURING 1975-1976

Station	Log HC/ml	Log HET/ml	Log HC/HET	Temp. (°C)	Salinity (ppt)	NO_3 (μgat/l)	PO_4 (μgat/l)	DOC (mg/l)	Log ALI (μg/l)
Fall 1975									
C1	2.6	5.0	-2.3	16.0	29.81	2.16	0.64	1.7	1.91
D1	0.4	4.0	-3.6	17.5	30.87	0.12	0.52	1.1	1.01
N3	-0.4	3.4	-3.7	17.0	32.39	2.29	0.32	1.0	-0.07
E3	-1.0	3.0	-4.0	14.4	32.96	1.12	0.34	0.9	1.79
F2	0.4	1.6	-1.3	20.5	35.24	0.20	0.09	1.4	1.44
J1	-2.5	1.4	-3.9	20.7	35.44	0.06	0.13	1.0	2.22
Winter 1976									
C1	1.3	4.4	-3.1	3.8	31.05	0.30	0.36	3.37	0.67
D1	1.0	4.0	-3.1	4.0	31.52	0.15	0.40	2.86	0.72
N3	-1.1	2.2	-3.3	5.5	32.54	0.03	0.26	1.95	0.90
E3	-0.9	1.4	-2.3	7.1	33.46	0.09	0.20	5.18	1.23
F2	1.2	2.6	-1.5	9.3	34.75	1.98	0.24	2.02	0.81
J1	-0.03	2.4	-2.4	8.4	34.89	0.05	0.04	3.94	0.76
Spring 1976									
C1	5.0	4.7	0.4	16.2	32.02	1.55	0.24	2.14	-0.23
D1	-1.6	4.4	-6.0	16.6	32.02	1.63	0.12	3.49	-0.43
N3	-0.03	3.0	-3.0	17.0	32.20	1.52	0.04	5.67	-0.61
E3	-1.6	2.2	-3.8	17.1	32.16	0.22	0.12	2.93	-0.43
F2	-1.6	3.0	-4.6	18.0	32.07	0.28	0.36	2.63	0.08
J1	-1.4	3.2	-4.5	17.8	31.78	0.09	0.04	2.22	-0.37
Summer 1976									
C1	3.0	4.9	-1.9	21.0	1.76	0.10	0.48	4.55	-0.37
D1	-0.4	3.0	-3.3	22.3	31.56	0.09	0.24	4.22	-0.81
N3	-0.4	2.9	-3.3	22.3	32.19	0.05	0.20	2.56	-0.85
E3	0.4	3.6	-3.3	21.1	31.10	0.08	0.16	4.12	-0.86
F2	-0.4	3.2	-3.5	21.0	33.91	0.10	0.04	2.06	-0.75
J1	-0.6	3.4	-4.0	20.5	33.93	0.10	0.04	2.46	-0.51

HC = Petroleum-degrading bacteria; HET = Heterotrophic bacteria; DOC = dissolved organic carbon; ALI = Dissolved aliphatic hydrocarbons.

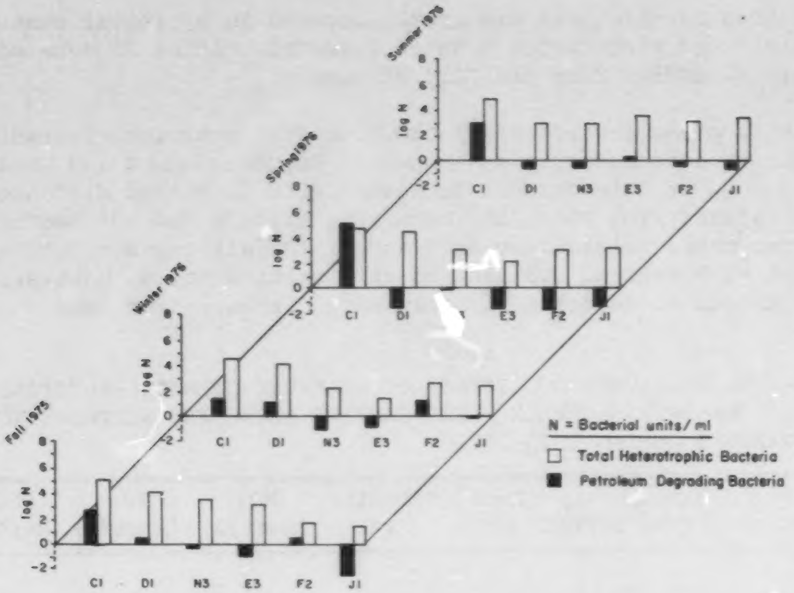

Figure 3. Viable counts of heterotrophic and petroleum-degrading marine bacteria in surface seawater samples (1 m) collected during 1975-1976.

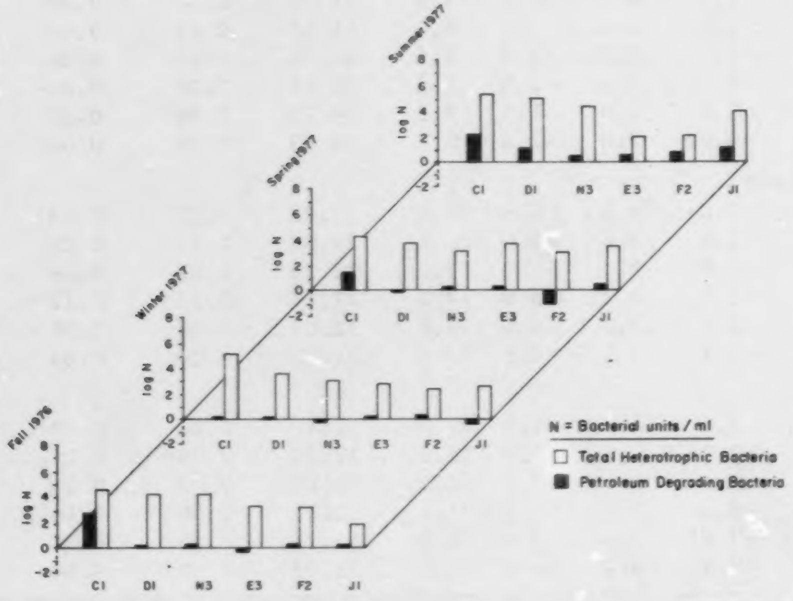

Figure 4. Viable counts of heterotrophic and petroleum-degrading marine bacteria in surface seawater samples (1 m) collected during 1976-1977.

TABLE 2. VIABLE BACTERIAL COUNTS AND VALUES OF SELECTED CHEMICAL-PHYSICAL PARAMETERS FOR ONE-METER SURFACE WATER SAMPLES ALONG THE SOUTHERN NEW JERSEY TRANSECT DURING 1976-1977

Station	Log HC/ml	Log HET/ml	Log HC/HET	Temp. (°C)	Salinity (ppt)	NO_3 (μgat/l)	PO_4 (μgat/l)	DOC (mg/l)	Log ALI (μg/l)	Log ATP (ng/l)
Fall 1976										
C1	2.8	4.6	-1.8	11.5	31.76	4.01	1.12	4.88	1.36	3.11
D1	0.1	4.2	-4.1	12.3	32.65	0.57	0.76	1.02	-0.34	2.83
N3	0.3	4.2	-3.9	12.6	32.79	0.27	0.40	3.05	-0.80	2.59
E3	-0.4	3.3	-3.6	13.1	33.05	N.A.	N.A.	3.43	-0.77	2.67
F2	0.3	3.2	-2.9	13.0	34.07	0.55	0.26	1.19	-0.92	2.59
J1	-3.2E-02	1.9	-1.9	15.6	35.44	1.65	1.08	7.90	-1.64	1.62
Winter 1977										
C1	-4.0E-03	5.1	-5.1	0.5	32.51	N.A.	N.A.	5.23	-1.33	2.89
D1	0.2	3.6	-3.5	2.8	34.39	0.80	0.26	3.20	N.A.	2.48
N3	0.2	3.0	-2.6	4.5	34.83	4.34	0.52	4.62	-0.85	2.57
E3	-0.2	2.8	-3.2	7.5	35.44	7.73	0.56	4.98	-1.02	2.69
F2	0.3	2.4	-2.0	9.6	35.44	11.60	0.76	8.00	-1.40	1.90
J1	-0.4	2.6	-3.0	10.5	34.83	4.34	1.11	1.95	-1.11	2.15
Spring 1977										
C1	1.3	4.1	-2.8	14.2	31.93	0.21	N.A.	3.90	-1.04	2.45
D1	-0.2	3.6	-3.8	13.5	33.23	N.A.	0.18	2.38	-1.21	2.43
N3	0.2	3.0	-2.8	14.3	33.39	0.02	0.22	2.42	-1.34	2.56
E3	0.3	3.5	-3.2	18.7	35.33	0.10	0.10	3.58	-1.44	2.11
F2	-1.1	2.9	-4.0	20.9	35.58	0.00	0.08	2.28	-1.47	2.36
J1	0.5	3.5	-2.0	20.5	36.03	0.02	0.00	1.85	-1.24	2.04
Summer 1977										
C1	2.1	5.3	-3.2	24.3	32.04	0.41	0.28	3.29	N.A.	3.08
D1	1.1	4.9	-3.8	25.4	32.11	0.18	0.04	6.37	N.A.	2.78
N3	0.5	4.4	-3.9	25.7	32.70	0.81	0.20	6.31	N.A.	2.45
E3	0.6	2.0	-1.4	25.6	32.03	0.13	0.16	6.04	N.A.	2.11
F2	0.8	2.1	-1.3	24.4	32.10	0.07	0.08	3.29	N.A.	2.18
J1	1.1	3.9	-2.8	26.6	35.56	0.81	0.32	4.70	N.A.	2.04

HC = Petroleum-degrading bacteria; HET = Heterotrophic bacteria; DOC = Dissolved organic carbon; ALI = Aliphatic hydrocarbons; N.A. = Not available.

TABLE 3. GEOMETRIC MEAN POPULATIONS OF PETROLEUM-DEGRADING (HC) AND HETEROTROPHIC (HET) MARINE BACTERIA OBTAINED FROM ONE-METER SURFACE WATER SAMPLES (1975-1976)

Bacterial Parameter	By season			
	Fall 1975	Winter 1976	Spring 1976	Summer 1976
log HC/ml	-0.1±1.7	0.2±1.1	-0.2±2.6	0.3±1.4
log HET/ml	3.0±1.4	2.8±1.2	3.4±1.0	3.5±0.7

	By station					
	C1	D1	N3	E3	F2	J1
log HC/ml	3.0±1.5	-0.2±1.1	-0.5±0.5	-0.8±0.8	-0.1±1.2	-1.1±1.1
log HET/ml	4.7±0.3	3.8±0.6	2.8±0.5	2.5±1.0	2.6±0.7	2.6±0.9

TABLE 4. GEOMETRIC MEAN POPULATIONS OF PETROLEUM-DEGRADING (HC) AND HETEROTROPHIC (HET) MARINE BACTERIA OBTAINED FROM ONE-METER SURFACE WATER SAMPLES (1976-1977)

Bacterial	By season			
	Fall 1976	Winter 1977	Spring 1977	Summer 1977
log HC/ml	0.4±1.1	0.02±0.3	0.2±0.8	1.0±0.6
log HET/ml	3.6±0.9	3.4 ±1.0	3.3±0.6	3.8±1.4

	By station					
	C1	D1	N3	E3	F2	J1
log HC/ml	1.6±1.1	0.3±0.5	0.2±0.3	0.2±0.4	0.1±0.8	0.3±0.6
log HET/ml	4.8±0.6	4.1±0.6	4.1±1.0	2.9±0.7	2.6±0.5	2.7±0.8

Viable counts of petroleum-degrading bacteria exhibited similar trends as counts decreased from inshore maxima of 10^5 bacterial units/ml to less than 3.2×10^{-3} bacterial units/ml at J1 during the fall of 1975. Seasonally, viable counts tended to be lowest during the winter inshore and highest during the summer. Viable counts along the transect tended to be related to water types. Thus, station C1, which generally exhibited the greatest populations of HC, was located in the coastal boundary layer. Stations D1, N3, and E3, which either exhibited lower or the lowest counts as one progressed seaward, were located in inner-outer shelf waters. Station F2 occasionally exhibited elevated values relative to E3 and J1. This station was frequently located in a convergence zone produced by the mixing of shelf and slope waters. Upwelling, associated with the convergence, apparently produced elevated biological activity owing to elevated inorganic nutrient levels.

Geometric mean and individual station count data indicated that the winter of 1977 was characterized by the lowest viable counts of petroleum-degrading bacteria. Generally, stations and seasons exhibiting counts with the largest standard deviations were those most strongly affected by changes in climate or hydrodynamic processes, i.e., least stable. Ratios of petroleum-degrading to "total" heterotrophs varied directly with viable counts of petroleum-degrading bacteria.

Physical and chemical parameters measured during this study are presented in Tables 1 and 2. Surface water temperatures generally increased with distance from land with the winter seasons illustrating the strongest temperature gradient. This was especially so during 1977 when the temperature at C1 was -0.5°C and pancake ice was present. Salinity also increased with distance from land. Levels of nitrates and phosphates did not vary with recognizable patterns with station but appeared to be greatest during the winter of 1977 when phytoplankton activity would be expected to be a minimum. As previously mentioned, elevated levels of nutrients were sometimes observed at the shelf break station F2. Slope water at J1 and intrusions of slope water further inshore generally resulted in the lowest values of inorganic nutrients.

Linear regression correlation coefficients were calculated for bacterial populations against various parameters (Tables 5 and 6). Significant positive correlation ($\alpha = 0.05$) was observed between petroleum-degrading bacteria and heterotrophic bacteria during 1975-76 and 1976-77. A significant ($\alpha = 0.05$) positive correlation between petroleum-degrading bacteria and the ratio of petroleum-degrading bacteria to "total" heterotrophs was noted. ATP concentrations, measured only during 1976-77, were significantly ($\alpha = 0.05$) correlated with "total" heterotrophic bacterial counts. Other significant ($\alpha = 0.05$) positive correlations were noted between petroleum-degrading bacteria and dissolved aliphatic hydrocarbons (1976-77) and between phosphate concentrations and viable bacterial counts (1975-76).

TABLE 5. LINEAR REGRESSION CORRELATION COEFFICIENTS FOR BACTERIA AGAINST SELECTED PARAMETERS SAMPLED DURING 1975-1976

	HC	HET	ALI	DOC	NO_3	PO_4
HC	--	0.65**	-0.14	0.08	0.32	0.49**
HET	0.65**	--	-0.27	0.06	0.35	0.55**
HC/HET	0.73**	0.03	-0.04	0.03	0.17	0.16

**Significant at $\alpha = 0.01$; HC--Petroleum-degrading bacteria; HET--Heterotrophic bacteria; ALI--Dissolved aliphatic hydrocarbons; DOC--Dissolved organic carbon.

Using closed flask systems, certain trends in viable counts of petroleum-degrading and "total" heterotrophic bacteria were observed with regularity. An example of these is shown in Figure 5. In all experimental treatments, levels of petroleum-degrading and heterotrophic bacteria increased from initial values with counts in enriched flasks being greater than either inoculated or control flasks. Elevated viable counts were maintained throughout the incubation period. The value of the ratio of petroleum-degrading to heterotrophic bacteria increased above initial values in all flasks within 3 d and was generally larger in flasks with oil.

TABLE 6. LINEAR REGRESSION COEFFICIENTS FOR BACTERIA AGAINST SELECTED PARAMETERS SAMPLED DURING 1976-1977

	HC	HET	ATP	ALI	DOC	NO_3	PO_4
HC	--	0.48*	0.34	0.55	0.22	-0.06	0.10
HET	0.48*	--	0.72**	0.41	-0.10	-0.14	-0.18
HC/HET	0.37	-0.61*	-0.48*	-0.01	0.22	0.11	0.20

*Significant at $\alpha = 0.05$; **Significant at $\alpha = 0.01$; HC--Petroleum-degrading bacteria; HET--Heterotrophic bacteria; ALI--Dissolved aliphatic hydrocarbons; DOC--Dissolved organic carbon.

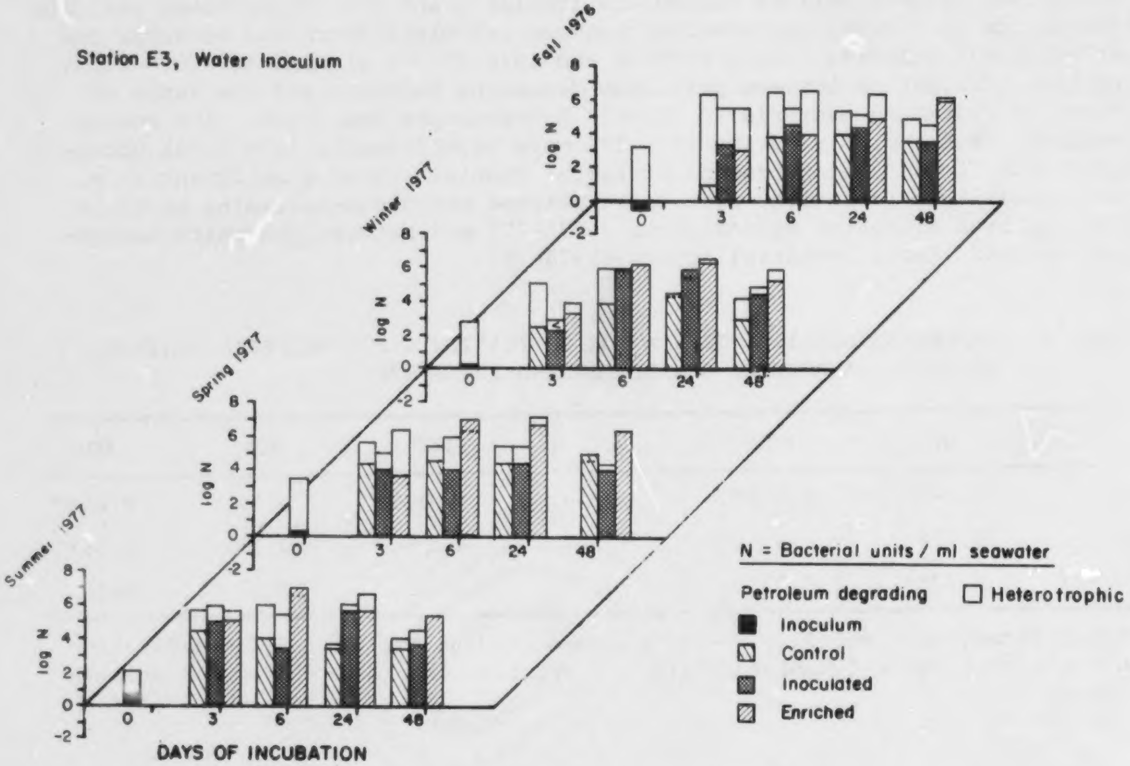

Figure 5. Typical changes observed in viable counts of heterotrophic and petroleum-degrading marine bacteria for closed flask petroleum degradation studies.

Degradation was assessed by disappearance of selected *n*-paraffins. Significant degradation, i.e., greater than 10% of the control summation of *n*-paraffins, was noted only in enriched flasks. In all cases, similar patterns of degradation, i.e., utilization of *n*-paraffins of short chain length prior to *n*-paraffins of longer chain lengths and isoprenoids, were observed. Rates of degradation and extent varied with season and inoculum source. Average rates of degradation were calculated based on the number of days of incubation required for degradation of 50% of the *n*-paraffins (Table 7). The latter were degraded most rapidly during the fall and slowest during the winter. Extent of degradation was defined as the maximum percentage of selected *n*-paraffins degraded during the incubation period (Table 8). Fall exhibited the greatest extent of degradation and winter the smallest. Despite the highest incubation temperature, experiments with summer inocula yielded relatively low rates and extent of degradation. With respect to inoculum source, stations C1 and N3 exhibited the greatest relative rates and extent of degradation. A consistent relationship between viable counts of petroleum-degrading bacteria and extent of degradation was not evident.

TABLE 7. DAYS OF INCUBATION REQUIRED FOR DEGRADATION[a] OF 50% OF *n*-ALKANES (nC_{10}-nC_{25}) IN CLOSED FLASKS FROM PETROLEUM DEGRADATION EXPERIMENTS

Station	Treatment[b]	Season			
		Fall 1976	Winter 1977	Spring 1977	Summer 1977
C1	Inoculated	NR	NR	NR	NR
	Enriched	48	48	48	48
D1	Inoculated	NR	NR	NR	NR
	Enriched	24	NR	NR	NR
N3	Inoculated	NR	NR	NR	NR
	Enriched	24	NR	48	24
E3	Inoculated	NR	NR	NR	NR
	Enriched	48	NR	48	NR
F2	Inoculated	NR	NR	NR	NR
	Enriched	48	NR	NR	NR
J1	Inoculated	NR	NR	NR	NR
	Enriched	NR	NR	48	NR
	Incubation Temperature (°C)	15	5	15	20

[a] Based on weight loss in treated flask as compared to control; [b] Inoculated = seawater + oil, Enriched = seawater + oil + nutrient amendment; NR = Degradation never reached 50%.

Results of viable count enumerations for the continuous dilution experiments are shown in Figure 6. Trends similar to those noted for closed flask systems were observed. Viable counts of petroleum-degrading and heterotrophic bacteria increased in both oil-free and oil-treated flasks. However, after this initial increase, viable counts of petroleum-degrading bacteria in oil-free flasks decreased to lower values than in oil-treated flasks. A relative lag in viable counts of petroleum-degrading bacteria in oil-treated flasks during the winter (6°C) was observed. Heterotrophic bacterial counts remained elevated in all flasks. Preliminary analysis of residual extracted oil proved disappointing as losses due to evaporation in the controls were equivalent to losses in the inoculated flasks.

TABLE 8. MAXIMUM AMOUNT OF DEGRADATION[a] OBSERVED DURING 48 DAYS OF INCUBATION

Station	Fall 1976	Winter 1977	Spring 1977	Summer 1977	Seasonal $\overline{X}$
C1	61.4	53.2	54.5	42.2	52.8
D1	63.3	24.0	0.0	40.4	31.9
N3	72.1	25.8	100.0	70.1	67.0
E3	51.2	33.9	50.7	14.2	37.5
F2	68.8	35.1	39.5	26.2	42.4
J1	25.8	36.4	54.1	26.2	35.6
All stations $\overline{X}$	57.1	34.7	49.8	36.6	

[a]Degradation as % $= 100 \left[1 - \dfrac{\Sigma\ \mu g\ n\text{-alkanes}\ (nC_{10}-nC_{25}),\ \text{Enriched Flask}}{\Sigma\ \mu g\ n\text{-alkanes}\ (nC_{10}-nC_{25}),\ \text{Control Flask}} \right]$

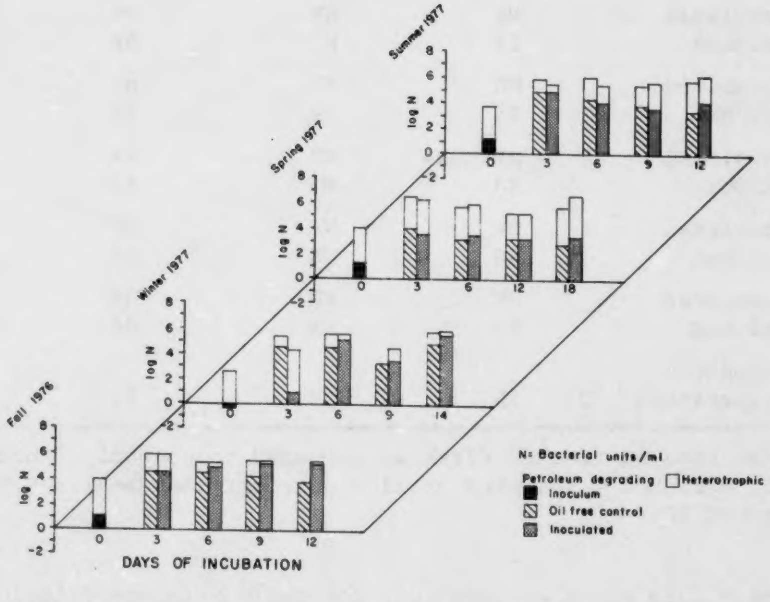

Figure 6. Changes in viable counts of heterotrophic and petroleum-degrading marine bacteria in "open" or continuous dilution petroleum degradation experiments.

DISCUSSION

The use of numbers of petroleum-degrading bacteria or the ratio of petroleum-degrading to "total" heterotrophic bacteria has been proposed as an indicator of the presence of petroleum or related products in aquatic environments (15). Laboratory studies have substantiated that in closed flask and simulated "open" systems, enrichment of petroleum-degrading bacteria results in elevation of the value of the ratio when hydrocarbons are present and adequate nutrients provided for their metabolism. Field data presented in this paper, obtained from seasonal monitoring of bacterial populations from surface waters of the MACS east of New Jersey, revealed that viable counts of petroleum-degrading bacteria (and the ratio of these to heterotrophs) did not consistently correlate with concentrations of dissolved aliphatic hydrocarbons, dissolved organic carbon, particulate organic carbon, nitrates, phosphates and ATP. Although a significant ($\alpha = 0.05$) correlation of petroleum-degrading bacteria with aliphatic hydrocarbons was observed for various seasons or for a single year (1976-1977), it is difficult to believe that a functional relationship underlies this response. More likely it is a reflection of correlation with some common variable. Although other investigators have reported significant quantitative relationships between petroleum-degrading bacterial counts and pollutant hydrocarbons (3, 15), the concentrations noted have generally been 1,000-10,000 times larger than those measured for most surface water MACS stations. Buckley et al. (1976) found no consistent relationship of viable counts with petroleum (as determined by fluorescence) concentrations in non-polluted estuarine waters.

Levels of petroleum-degrading bacteria appeared to be related to water type. Thus, the largest viable counts were usually measured in the coastal boundary layer, a water type characterized by proximity to seasonal marina activities and generally the highest levels of nutrients, organic carbon and aliphatic hydrocarbons. Lower values of petroleum-degrading bacteria were usually found for the remaining inner (D1, N3) and outer (E3) shelf stations. During six seasons relatively elevated viable counts were measured in the vicinity of the shelf break (F2). Here shelf and slope water mixed and the resulting convergence with associated upwelling would appear to stimulate biological activities. Finally, the lowest viable counts measured usually were found beyond the shelf break seaward of the frontal zone (J1) in "oceanic" or Gulf Stream eddy water. Simple correlations of viable bacterial counts with physical-chemical data may not be possible considering the dynamic nature of the MACS shelf-slope hydrographics. Considering the uncertainties associated with a data base limited to several samples/station each season, the precision of the chemical analyses, and the large standard deviations implicit in the MPN technique utilized, the lack of consistent linear correlations was not surprising. However, despite these difficulties, basic trends in the data were observed and indicated that petroleum-degrading bacteria tended to decrease with distance from shore with variations on this pattern being due to interactions of shelf-slope water types.

Although petroleum-degrading bacteria could be detected at all stations, absolute numbers of bacteria were not always indicative of the in-vitro degradation potential in closed flask experiments. With nutrient enrichment all stations could supply an inoculum with the capacity to degrade some proportion of the *n*-paraffins present. Those stations exhibiting the greatest aliphatic degradation potential may have been pre-adapted through selection of those genera capable of degrading petroleum. This adaptability, however, was not consistently reflected in viable surface water counts.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. American Public Health Association. 1976. Standard methods for the examination of water and wastewater. 14th ed. American Public Health Association, Inc., Washington, D.C.

2. Atlas, R. M., and R. Bartha. 1973. Fate and effects of polluting petroleum in the marine environment. Residue Rev. 49:49-85.

3. Atlas, R. M., and R. Bartha. 1973. Abundance, distribution, and oil biodegradation potential of microorganisms in Raritan Bay. Environ. Pollut. 4:291-300.

4. Buckley, E. N., R. B. Jonas, and F. K. Pfaender. 1976. Characterization of microbial isolates from an estuarine ecosystem: relationship of hydrocarbon utilization to ambient hydrocarbon concentrations. Appl. Environ. Microbiol. 32:232-237.

5. Gunkel, W. 1968. Bacteriological investigations of polluted sediments from the Cornish coast following the Torrey Canyon disaster, p. 151-158. *In* E. B. Colwell (ed.), The biological effects of oil pollution on littoral communities.

6. Kator, H., and R. Herwig. 1977. Microbial responses after two experimental oil spills in an Eastern coastal plain estuarine ecosystem, p. 517-522. *In* Proc. API-EPA-USCG Oil Spill Conf., New Orleans, La.

7. Lewin, R. A. 1974. Enumeration of bacteria in seawater. Int. Rev. Hydrobiol. 59:611-619.

8. McNair, H. M., and E. J. Bonnelli. 1969. Basic gas chromatography. Consolidated Printers, Berkeley, California.

9. Oppenheimer, C. H., W. Gunkel, and G. Gassmann. 1977. Microorganisms and hydrocarbons in the North Sea during July-August 1975, p. 592-609. *In* Proc. API-EPA-USCG Oil Spill Conf., New Orleans, La.

10. Pritchard, P. H., and T. J. Starr. 1975. The microbial degradation of oil in continuous culture. Office of Naval Research, Task No. NR 133-070. Annual Report No. 2.

11. Seki, H. 1976. Method for estimating the decomposition of hexadecane in the marine environment. Appl. Environ. Microbiol. 31:439-441.

12. Strickland, J. D. H., and T. R. Parsons. 1972. A practical handbook of seawater analysis. Bull. 167, Fish. Res. Bd. Canada, Ottawa.

13. Virginia Institute of Marine Science. 1978. Middle Atlantic Outer Continental Shelf environmental studies. Vol. II, Chemical and biological benchmark studies. Prepared by VIMS, Gloucester Point, Virginia, under contract No. 08550-CT-5-42 with the Bureau of Land Management, U.S. Department of Interior (in press).

14. Virginia Institute of Marine Science. 1977. Middle Atlantic Outer Continental Shelf environmental studies. Vol. II, Chemical and biological benchmark studies. Prepared by VIMS, Gloucester Point, Virginia, under contract No. 08550-CT-5-42 with the Bureau of Land Management, U.S. Department of Interior. 1450 pp.

15. Walker, J. D., and R. R. Colwell. 1976. Enumeration of petroleum-degrading microorganisms. Appl. Environ. Microbiol. 31:198-207.

DISTRIBUTION AND DEGRADATIVE POTENTIAL OF KEPONE-RESISTANT BACTERIA IN THE JAMES RIVER AND UPPER CHESAPEAKE BAY

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ABSTRACT

Kepone-resistant bacteria were isolated from the James River, Baltimore Harbor, and Upper Chesapeake Bay where their numbers reflected the degree of anthropogenic contamination at the sampling sites. Gram negative bacteria, of which the majority were *Pseudomonas* spp., represented approximately 99% of the Kepone-resistant bacteria capable of growth on 100 ppm Kepone agar. Screening of nine Kepone-resistant isolates from Baileys Creek revealed the presence of large molecular weight plasmids, and curing one of the isolates with mitomycin C rendered it sensitive to Kepone. A 9-chloro-Kepone derivative, observed by electron capture gas-liquid chromatography, was found to be associated with microbial activity in Kepone-enriched Baileys Creek sediment.

Within the past ten years the James River and Chesapeake Bay have been contaminated with Kepone, a chlorinated insecticide which is a chemically and physically stable compound. Surveys of these regions have revealed the presence of Kepone in finfish taken from areas beyond the James River and Chesapeake Bay. Like many other chlorinated insecticides, Kepone has been found to induce toxic effects in higher organisms, e.g., scoliosis in fish (11).

Until recently, research on Kepone concerned invertebrate toxicity (12, 14, 20), effects on soil microorganisms (12) and bactericidal effects (4). Block (4, 5), finding Kepone to be very effective in inhibiting growth of Gram positive organisms at concentrations as low as 8 ppm, proposed the use of Kepone as an antimicrobial agent. Other, more recent work by Bourquin et al. (6) and Brown et al. (7) has demonstrated toxic effects of Kepone on estuarine microorganisms. There is a lack of information on biodegradation, especially potential degradation of Kepone by bacteria in estuarine and freshwater environments.

The purpose of this study was to determine the distribution of Kepone-resistant bacteria in the James River and Upper Chesapeake Bay, and to study the physiology and taxonomy of these bacteria. Identification of the genetic basis of Kepone resistance in the bacterial isolates was also of interest, as was the degradative potential of bacterial isolates obtained from enrichment cultures.

MATERIALS AND METHODS

Samples of water and sediment from stations in the James River, Baileys Creek, Baltimore Harbor and Eastern Bay of the Chesapeake Bay were collected over a period of 18 months. Five stations in the James River, including Baileys Creek, were sampled at established intervals (Fig. 1). Water samples were collected aspetically at a one-meter depth using a Niskin sampler (General Oceanics, Miami, Fla.) and sediment samples with a Ponar Grab (Wildco, Saginaw, Mich.).

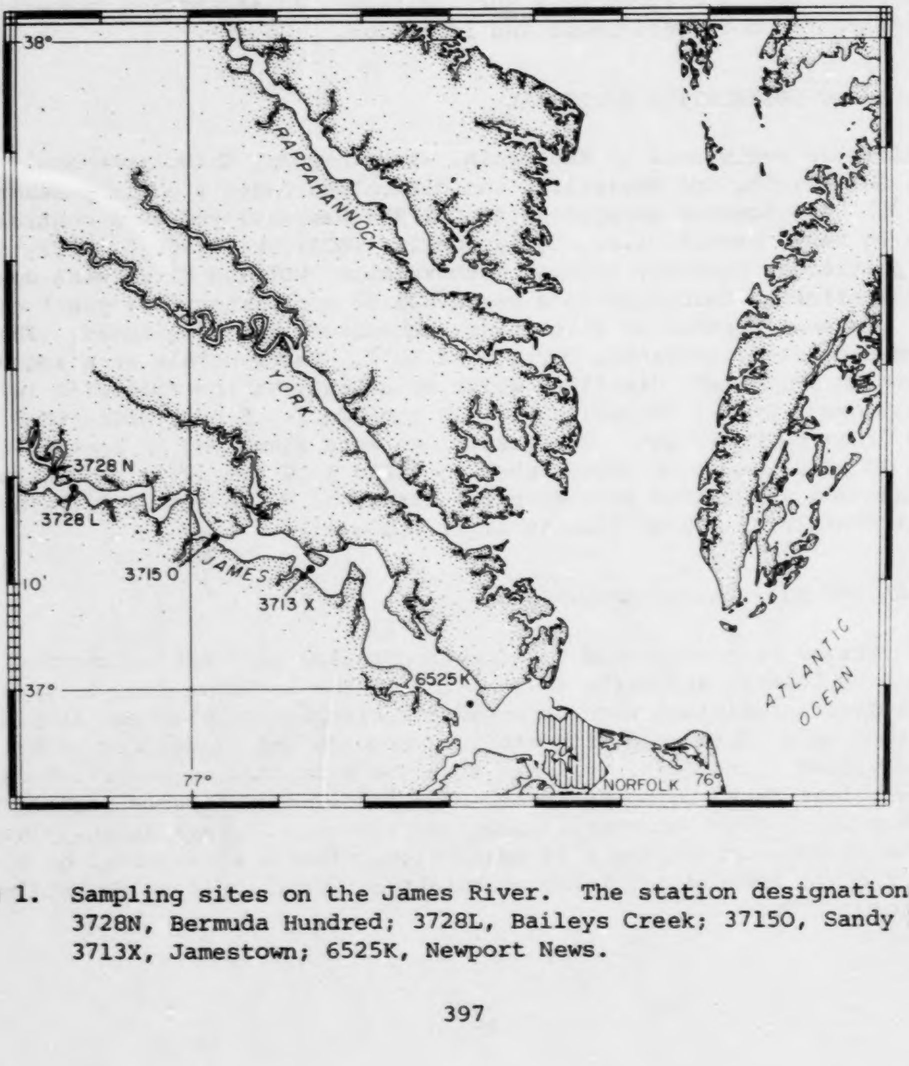

Figure 1. Sampling sites on the James River. The station designations are: 3728N, Bermuda Hundred; 3728L, Baileys Creek; 3715O, Sandy Point; 3713X, Jamestown; 6525K, Newport News.

ISOLATION

Samples were inoculated into yeast extract-minimal salts broth containing 100 ppm Kepone to obtain Kepone-resistant bacteria. Enumeration of total, viable heterotrophs and of Kepone-reistant bacteria was accomplished using the spread plate method. UBYE agar (19), a medium described elsewhere and containing appropriate salts, was employed with Kepone in acetone added as a surface film to the solidified agar to give a final concentration (w/v) of 100 ppm. Kepone-resistant, facultative anaerobes were enumerated using identical media incubated in Gas-Pak containers (Baltimore Biological Laboratory) for one week. PCB-resistant organisms were enumerated on UBYE agar to which 100 mg/liter Aroclor 1254 had been added. Oil-degrading organisms were enumerated on Oil Agar #2 (24). Total coliforms were determined following Standard Methods published by the American Public Health Association (21).

Kepone-resistant organisms were isolated and maintained on media containing 0.1% yeast extract, 0.1% proteose peptone, 0.02% K_2HPO_4, 0.02% KH_2PO_4, 0.02% $NaNO_3$, 0.02% $(NH_4)_2HPO_4$ and 2% Bacto-agar, with 0.1 ml of a 10 mg/ml Kepone acetone solution added as a surface film. An incubation temperature of 30°C was used for both enrichment and isolation.

RESISTANCE AND DEGRADATIVE POTENTIAL

Antibiotic resistance to Kanamycin, Streptomycin, Chloramphenicol, Tetracycline, Gentamicin, and Ampicillin was determined using the Kirby-Bauer method (3). *Pseudomonas aeruginosa* ATCC 27853 was employed as a control. Resistance to heavy metals, i.e., $HgCl_2$, $CdCl_2$, $ZnCl_2$, $NiCl_2 \cdot 6H_2O$, $CrCl_3 \cdot 6H_2O$, and the pesticides lindane, aldrin, methoxychlor, DDT and Mirex were determined by monitoring turbidity in a broth medium containing 0.1% yeast extract and 0.1% proteose peptone to which these substances had been added. The tubes were examined after incubation for 4 d at 30°C. Heavy metals were added to the medium as deionized, distilled water solutions, at the following (w/v) concentrations: $ZnCl_2$, 50 ppm; $HgCl_2$, 10 ppm; $CdCl_2$, 5 ppm; $NiCl_2 \cdot 6H_2O$, 10 ppm; and $CrCl_3 \cdot 6H_2O$, 10 ppm. The pesticides were dissolved in acetone and added in 10 µl aliquots to broth tubes to yield a 10 ppm (w/v) concentration. Oil degradation was tested following the method of Mills et al. (17), using MMC broth containing 0.1 ml Arabian light crude oil.

IDENTIFICATION AND PLASMID ANALYSIS

All strains were subjected to a testing regime of "core" characteristics, as defined by Colwell and Wiebe (10). Nine of the isolates from Baileys Creek that were Kepone-resistant were screened for plasmids by electrophoresis in 0.7% agarose gels, developed with ethidium bromide and visualized under longwave ultraviolet light (16). Plasmid size was estimated by comparison with two marker plasmids harbored by *Escherichia coli* J53 (reference strains received from Dr. Esther Lederberg); S-a, containing a 25 mega dalton plasmid, and strain J53RP4, containing a 34 mega dalton plasmid as reported by S. Falkow. Curing was carried out in an overnight BHI broth culture containing 0.1 µg/ml mitomycin C.

DEGRADATION OF KEPONE

Utilization of Kepone as a sole carbon source was determined by inoculation of a minimal salts broth, supplemented with Kepone in concentrations of 1, 10, 100, 500, and 1000 ppm, with 0.1 ml of washed log-phase cells. Inoculated media was incubated at 30°C, in the dark, for one, six and 12 months.

Microbial degradation of Kepone was examined using homogenized sediment collected from the top 30 cm of the bottom of the James River at a site located at the mouth of Baileys Creek. One set of three 500-ml Erlenmeyer flasks received 200 g.w.w. (gram wet weight) each of autoclaved, air-dried sediment, and a second set received an equal amount of non-sterile sediment. A third set of flasks containing non-sterile sediment were not amended with Kepone. All flasks were brought to equal moisture content, i.e., each flask of sterile, air-dried sediment received distilled water to achieve a moisture content equivalent to the in-situ, non-sterile sediment, with deionized, distilled water "spiked" with 30 μg analytical Kepone/100 g.w.w. sediment and incubated in the dark at 30°C for 8 weeks. Concentration of background Kepone in the homogenate prior to enrichment was determined by extraction of triplicate 100 g samples, followed by gas-liquid chromatography of the extracts.

Extraction of sediment for Kepone analysis was carried out using overnight Soxhlet extraction of air-dried, sieved samples with 200 ml of 1% methanol-benzene. The solvent extract was concentrated to ca 5 ml on a rotary flash-evaporator (Büchi, Brinkmann Instruments, Westbury, New York) and cleaned on a column of 6.0 g activated Florisil PR, 60/100 mesh, topped with 2.0 g anhydrous sodium sulfate. After the column was prewetted with 1% methanol-4% benzene-hexane, the Kepone was eluted in three fractions, the first eluting with 1% methanol-4% benzene-2% acetonitrile in hexane. The three fractions were combined and concentrated to ca 2.0 ml on a rotary flash-evaporator and added to a calibrated vial. The concentrate was evaporated to dryness under a stream of nitrogen at 40°C and resuspended in 1% methanol-benzene for gas-liquid chromatography. Solvent controls were analyzed as described above. Extraction efficiency for spiked sediment samples yielded recoveries of 85.6-97.1%. Values reported are corrected for extraction efficiency.

Gas-liquid chromatographic analysis was performed on a Shimadzu GC-4BM equipped with a ^{63}Ni electron capture detector and a 3 m x 3 mm column of 1.5% OV-17/1.95% QF-1 on 80/100 Chromosorb WHP. Column temperature was held isothermal at 230°C with a N_2 flow rate of 30 ml/min, and detector temperature of 250°C. Kepone concentration was determined from the calibration curve for peak-area measurements of Kepone (8, 18, 22). Kepone standards were run after every third sample.

CHEMICALS

Technical grade Kepone was donated by Allied Chemical Corporation, Morristown, N.J., and determined to be 88.6% pure. Analytical grade Kepone was obtained by recrystallization in toluene, and its purity confirmed by GLC analysis. All solvents were pesticide-grade quality (Burdick and Jackson, Muskegon, Michigan).

RESULTS AND DISCUSSION

Microbial populations in the Chesapeake Bay and James River water and sediment were enumerated during seven cruises between 1976 and 1977. Three of these cruises, conducted in 1977, are reported here.

Numbers of Kepone-resistant bacteria were found to decrease with distance downstream from Hopewell, Virginia, the major impact site of Kepone contamination. The percentage of total viable, aerobic bacteria in the water column, that were Kepone-resistant, fluctuated but remained constant in the sediment. The number of total coliforms was, in general, high in both water and sediment, notably at Baileys Creek in the James River where the numbers of Kepone-resistant bacteria were significantly large (Table 1).

It was possible to compare the numbers of Kepone-resistant bacteria in Colgate Creek and Bear Creek in Baltimore Harbor, and at the Chester River and Eastern Bay stations, with results for samples collected in Baileys Creek and Bermuda Hundred in the James River. Water and sediment counts for both of the James River stations were significantly higher than for the Upper Chesapeake Bay stations. In general, bacterial counts for samples collected at the unpolluted stations, i.e., Chester River and Eastern Bay, were lower than for samples collected in Baltimore Harbor. The latter were less than the counts obtained at the James River stations. These relationships were strongly evident for the sediment samples (Table 2).

The total numbers of facultative anaerobes resistant to Kepone were largest at the 20 to 30 cm depth in sediment cores taken at the Windmill Point channel at Baileys Creek. However, the total number of facultative anaerobes that were Kepone resistant amounted to less than 2% of the total microbial populations (Table 3).

The bacteria comprising the microbial populations in the Upper Chesapeake Bay did not demonstrate a marked capability for growth in the presence of chlorinated hydrocarbons, in general, compared with a significant capability for petroleum degradation. Less than 2% of the bacteria comprising the total viable aerobic heterotrophic bacterial populations in the samples collected in Baltimore Harbor were resistant to 100 ppm Kepone and less than 10% were resistant to 100 ppm Aroclor 1254. In contrast, ca 16% of the bacteria present in the water column and 37% of those in the sediment, as enumerated by plate counts on oil agar, were capable of degrading petroleum (Table 4).

In order to study the taxonomy and physiology of Kepone-resistant bacteria, colonies were transferred from UBYE agar medium containing 100 ppm Kepone to fresh UBYE-Kepone agar for purification. Enrichment for Kepone-resistant bacteria in Baileys Creek sediment and water, using flasks to which had been added 1 mg/100 g.w.w. or ml Kepone, was made by successive transfers to liquid medium containing Kepone, whereupon all cultures, including isolates from the plate counts, were tested for growth in 1000 ppm Kepone broth. All of the strains were capable of growth in the medium containing 1000 ppm Kepone. Therefore, all strains were concluded to be resistant to Kepone.

TABLE 1. MICROBIAL POPULATIONS IN SAMPLES COLLECTED AT JAMES RIVER STATIONS[a]

Station	Water				Sediment			
	TVC	Kepone[r]	% Kepone[r]	Total coliforms MPN/100 ml	TVC	Kepone[r]	% Kepone[r]	Total coliforms MPN/100 ml
Bermuda Hundred-3728N	1.2×10^6	5.7×10^3	0.48	1.6×10^2	9.7×10^6	3.6×10^6	37	2.4×10^5
Baileys Creek-3728L	8.4×10^6	3.4×10^5	6.1	1.7×10^4	8.2×10^6	5.9×10^5	7.2	2.5×10^5
Sandy Point-37150	6.9×10^3	2.4×10^3	35	7.0×10^1	3.6×10^6	2.9×10^5	8.2	7.0×10^3
Jamestown-3713X	5.6×10^3	8.6×10^2	15	3.4×10^1	4.1×10^6	1.0×10^5	2.4	2.5×10^4
Newport News-6525K	5.9×10^3	5.6×10^2	9.5	8.0	4.3×10^5	2.3×10^4	5.3	9.0

[a] Mean plate counts from three sampling expeditions, 1977.

[r] Denotes resistance.

TABLE 2. NUMBERS OF KEPONE-RESISTANT BACTERIA AT CHESAPEAKE BAY, BALTIMORE HARBOR AND JAMES RIVER STATIONS[a]

Station location	Water	Sediment
Chester River	5.7×10^2	5.8×10^3
Eastern Bay	$<10^1$	1.4×10^3
Colgate Creek	1.3×10^4	7.5×10^4
Bear Creek	4.1×10^4	7.2×10^4
Bermuda Hundred	5.7×10^3	3.6×10^6
Baileys Creek	3.4×10^5	5.9×10^5

[a] Determined from mean plate counts for three sampling expeditions.

TABLE 3. DISTRIBUTION OF KEPONE-RESISTANT AEROBIC AND FACULTATIVE ANAEROBIC BACTERIA IN SEDIMENT CORES COLLECTED AT THE WINDMILL POINT CHANNEL

Depth (cm)	Total heterotrophic aerobes (CFU/g.w.w. $\times 10^4$)	Total facultative anaerobes (CFU/g.w.w. $\times 10^2$)	Ratio of anaerobe/aerobe (CFU/g.w.w. $\times 10^{-3}$)
Surface	72	5.5	0.08
10	32	6.6	0.21
20	6.2	10	1.61
30	6.5	10	1.54
40	26	6.1	0.24

TABLE 4. MEAN PERCENTAGES OF TOTAL VIABLE HETEROTROPHIC POPULATIONS IN BALTIMORE HARBOR

	Kepone	PCB-1254	Oil Agar #2
Top water	0.45	3.0	16
Bottom water	1.31	--	--
Sediment	2.0	9.7	37

Taxonomic analysis of 88 of 300 isolates revealed approximately 99% to be Gram negative, oxidative rods. Further testing of 50 of the isolates to date revealed 36 to be *Pseudomonas* spp., 6 *Aeromonas hydrophila*, 5 *Flavobacterium* spp., and 3 *Acinetobacter* spp., using "core" characteristics for identification. Many of the 88 strains were lipolytic and proteolytic, but not amylolytic. A majority were denitrifiers and hydrocarbonoclastic organisms and a smaller number were proteolytic strains (Table 5).

TABLE 5. PHYSIOLOGICAL CHARACTERISTICS OF KEPONE-RESISTANT BACTERIA[a]

Source	Lipolysis	Starch hydrolysis	Proteolysis	Denitrification	Oil degradation
James River	48	1.1	38	77	69
Baltimore Harbor	55	40	15	55	80

[a] Values represent percentage of all Kepone-resistant isolates from James River or Baltimore Harbor that possess the characteristics listed.

Six isolates from Baileys Creek, all *Pseudomonas* spp., were found to possess one or two large plasmids, 25 or 45 million daltons in size, by agarose gel electrophoresis. Plasmids were not detected in three of the isolates tested. The nine strains were all subjected to a set of physiological tests, including resistance to antibiotics, heavy metals, and pesticides. One of the plasmid-carrying strains was found to be resistant to ampicillin, all of the pesticides and heavy metals tested and able to degrade oil. Curing the strain by mitomycin C rendered it sensitive to Kepone, but did not alter its resistance patterns to antibiotics, heavy metals or pesticides. None of the physiological characteristics of the cured isolate, when retested after curing, differed from those of the parental strain. The evidence to day suggests that in this isolate, the plasmid only conveys resistance to Kepone.

In degradation studies employing Kepone-resistant strains, no turbidity or increase in cell numbers was detected in any broth cultures when Kepone was provided as the sole source of carbon. Growth was obtained if a supplemental carbon source was provided.

In another study fresh samples of Baileys Creek sediment were placed in flasks with 30 μg/100 g.w.w. Kepone and incubated for 8 weeks at 30°C in the dark. No other nutrients were added. Gas-liquid chromatographic analyses of Kepone-enriched sediments to detect degradation were run in triplicate and corrected for background Kepone contamination, which amounted to 2.2 ppm Kepone. Analysis of the Kepone-enriched and non-amended control flasks revealed only Kepone to be present (Fig. 2).

The non-sterile enriched flasks, however, exhibited a major peak, in addition to the Kepone peak (Fig. 3). Analytical evaluation of Kepone and Mirex photoproducts by Alley et al. (1, 2), Ivie et al. (15) and Carlson (9) indicated that the behavior of the monohydro forms of Kepone was the same as that for the secondary peak noted in this study. The concentration of the Kepone derivative was less than 0.1% of the total Kepone added, as determined by peak-area comparison with calibration curves of Kepone. A similar derivative has also been reported by Dr. James Smith of Allied Chemical Corporation (personal communication), who analyzed soil heavily contaminated with Kepone. Confirmation of the identity of the derivative by mass spectrometry is in progress (23), with the analysis being done in collaboration with Dr. Henry Fales of the National Institutes of Health.

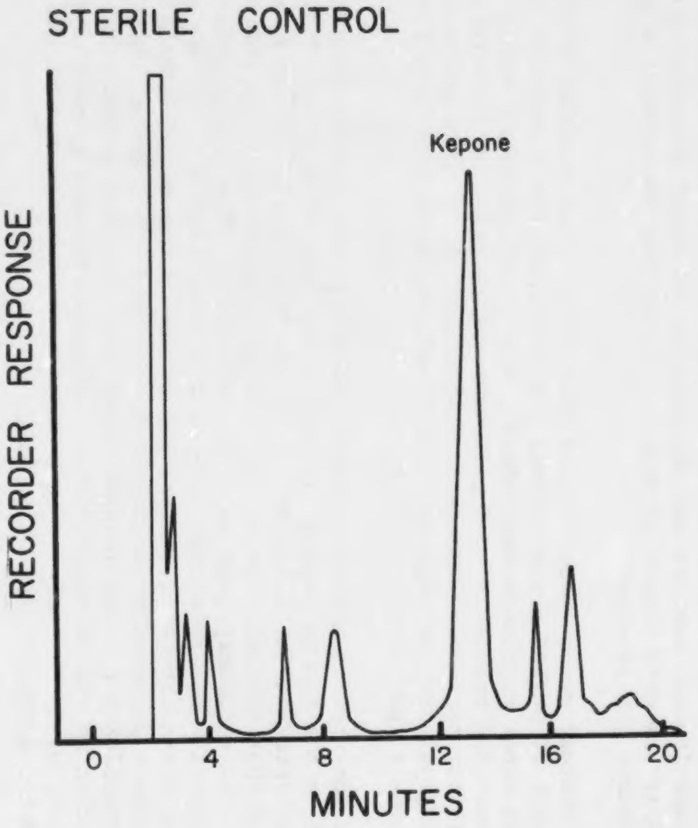

Figure 2. Electron capture detector GLC tracing of sterile (chemical control) Kepone-enriched controls.

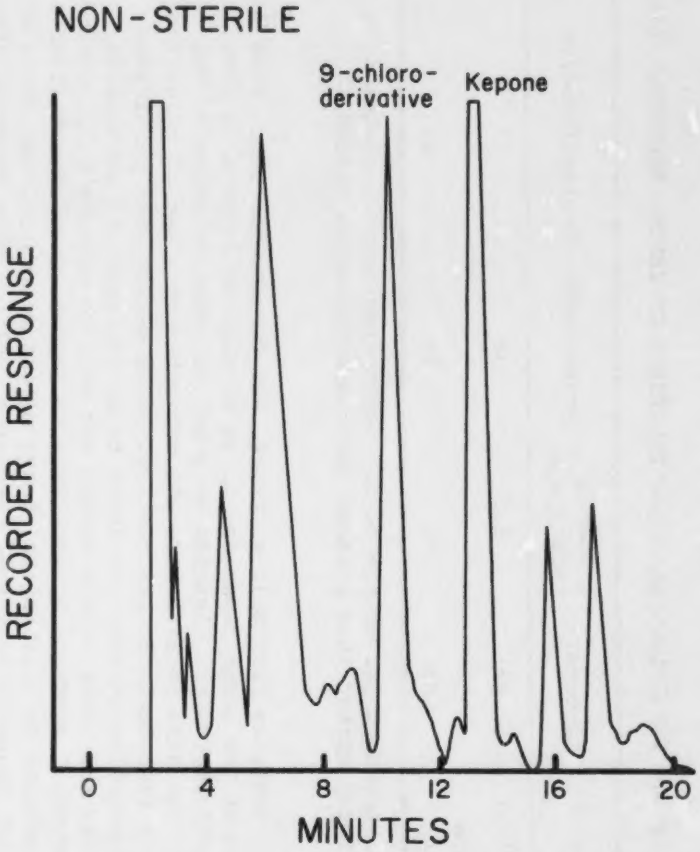

Figure 3. Electron capture detector GLC tracing of non-sterile (biological treatment) Kepone-enriched flasks.

Thus, by enumeration of the various physiological groups of microorganisms found in the Chesapeake Bay and James River, it is concluded that microbial resistance to Kepone can be demonstrated for several genera of estuarine and freshwater bacteria. This resistance occurs predominantly in Gram negative bacteria, particularly *Pseudomonas* spp., which appear to be physiologically diverse. Microbial strains resistant to Kepone were widely distributed throughout the Upper Chesapeake Bay and James River, but were present in largest numbers in the James River and Baltimore Harbor where pollution is a problem and the total viable aerobic heterotrophic bacterial populations are very large, compared to other parts of the Bay. The percentage of Kepone-resistant bacteria comprising the total, viable, aerobic heterotrophic microbial populations in the James River remained constant in the sediment, ca 6%, but fluctuated between 1-35% in the water column. This situation possibly arises from the presence of given bacterial species present in relatively stable numbers in the sediment, with fluctuations in the overlying water in response to abiotic or anthropogenic factors. Despite the fact that a very large percentage of the microbial population in Baltimore Harbor was able to degrade petroleum, relatively few of the strains were resistant to Aroclor 1254 and Kepone, i.e., 1-10% of the total population.

ACKNOWLEDGMENTS

L. A. McNicol (Department of Microbiology, University of Maryland) analyzed and identified the Kepone resistance plasmids. We thank D. C. Wolf for his advice and review of this manuscript. We also thank the Captain and crew of the R/V *Aquarius* and the R/V *Orion* of the Chesapeake Biological Laboratory for their assistance in collection of the samples.

LITERATURE CITED

1. Alley, E. G., D. A. Dollar, B. R. Layton, and J. P. Minyard, Jr. 1973. Photochemistry of Mirex. J. Agric. Food Chem. 121:138-139.

2. Alley, E. G., B. R. Layton, and J. P. Minyard, Jr. 1974. Identification of the photoproducts of the insecticides Mirex and Kepone. J. Agric. Food Chem. 22:442-445.

3. Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Twick. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45:493-496.

4. Block, S. S. 1968. Antibacterial and antifungal activity of Kepone derivatives. Dev. Ind. Microbiol. 9:430-436.

5. Block, S. S., and M. M. Darley. 1969. Kepone as bactericides. Allied Chemical Corp., U.S. 3,448,194 (Cl. 313-424; A61k).

6. Bourquin, A. W., S. K. Alexander, H. K. Sp-idel, J. E. Mann, and J. F. Fair. 1972. Microbial interactions with cyclodiene pesticides. Dev. Ind. Microbiol. 13:264-276.

7. Brown, L. R., G. W. Childers, and D. D. Vaishnav. 1974. Effects of Mirex and related compounds on estuarine microorganisms. Dev. Ind. Microbiol. 16:268.

8. Burke, J. A., and W. Holswade. 1965. Gas chromatographic column for pesticide residue analysis; retention time and response data. J. Assoc. Off. Agric. Chem. 49:374-385.

9. Carlson, D. A., K. D. Konyha, W. B. Wheeler, G. P. Marshall, and R. G. Zaylskie. 1976. Mirex in the environment: its degradation to Kepone and related compounds. Science 194:939-941.

10. Colwell, R. R., and W. J. Wiebe. 1970. "Core" characteristics for use in classifying aerobic, heterotrophic bacteria by numerical taxonomy. Bull. Georgia Acad. Sci. 28:165-185.

11. Couch, J. A., J. T. Winstead, and L. R. Goodman. 1977. Kepone-induced scoliosis and its histological consequences in fish. Science 197:585-587.

12. de la Cruz, A. A., and S. M. Naqui. 1973. Mirex incorporation in the environment: uptake in aquatic organisms and effects on the rates of photosynthesis and respir [illegible] Arch. Environ. Contam. Toxicol. 1:255-264.

13. Gawaad, A. A., M. Hammad, and F. H. El-Gayar. 1973. Effect of some soil insecticides on soil microorganisms. Agrokem. Talajtan 22:161-168.

14. Hansen, D. J., L. R. Goodman, and A. J. Wilson, Jr. 1977. Kepone chronic effects on embryo, fry, juvenile, and adult sheepshead minnows (*Cyprinodon variegatus*). Chesapeake Sci. 18:227-232.

15. Ivie, G. W., H. W. Dorough, and E. G. Alley. 1974. Photodecomposition of Mirex on silica gel chromatoplates exposed to natural and artificial light. J. Agric. Food Chem. 22:933-935.

16. Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127:1529-1537.

17. Mills, A. L., C. Breuil, and R. R. Colwell. 1978. Enumeration of degrading marine and estuarine microorganisms by the Most Probable Number Method. Can. J. Microbiol. 24:552-557.

18. Moseman, R. F., H. L. Crist, T. R. Edgerton, and M. K. Ward. 1977. Electron capture gas chromatographic determination of Kepone residues in environmental samples. Arch. Environ. Contam. Toxicol. 6:221-231.

19. Sayler, G. S., J. D. Nelson, Jr., A. Justice, and R. R. Colwell. 1975. Distribution and significance of fecal indicator organisms in the Upper Chesapeake Bay. Appl. Microbiol. 30:625-638.

20. Schimmel, S. C., and A. J. Wilson, Jr. 1977. Acute toxicity of Kepone to four estuarine animals. Chesapeake Sci. 18:224-227.

21. Standard Methods for the Examination of Water and Wastewater. 1971. American Public Health Association, Washington, D.C.

22. Thompson, J. F. 1968. Retento-Rule, a device for rapid peak identification of chlorinated pesticides. J. Gas Chromat. 6:560-562.

23. Uk, S., C. M. Himel, and T. F. Dirks. 1972. Mass spectral pattern of Mirex and of Kepone and its application to residue analysis. Bull. Environ. Contam. Toxicol. 7:207-214.

24. Walker, J. D., and R. R. Colwell. 1975. Factors affecting enumeration and isolation of actinomycetes from Chesapeake Bay. Mar. Biol. 30:193-201.

DISCUSSION

CONTRIBUTED SESSION

TIEDJE: I would like to compliment Lilly Young and associates. I thought that was an outstanding advancement in the understanding of anaerobic degradation of aromatic compounds. One thing that was very interesting to me is the apparent degradation of the aromatic ring by the primary organism without the production of hydrogen. In other words, you do not need the hydrogen sink to simulate the degradation. Is that a correct interpretation?

YOUNG: We did not monitor hydrogen in our systems, because it is utilized very rapidly. In that last slide where we inhibited the system, we were surprised to find that an active electron sink was not needed for benzoate to be utilized. It doesn't seem that ferulate-utilizing organisms need that electron sink for continual ferulic acid decomposition. I would think the next experiment to do would be to look for an increase in hydrogen in those experiments.

TIEDJE: It would mean also that the organism should be easily isolated.

YOUNG: Yes, in terms of separating the system, the ferulate-utilizing organism should be very easy to pull out.

TIEDJE: Have you gone through the thermodynamic calculations on whether it's feasible to have degradation without a hydrogen sink?

YOUNG: No. We gathered some thermodynamic information on ferulate, but our data are incomplete.

COONEY: Dr. Young, what was the solid phase or the sediment phase, say, in your cultures?

YOUNG: We have sodium sulfide as a reducing agent with iron as ferric chloride; so it's a ferrous sulfide precipitate in our systems which helps keep the system reduced.

DiGERONIMO: Dr. Young, I would like to go along with Dr. Tiedje and congratulate you on the work. Maybe I'm reading your stoichiometry wrong, but regarding your anaerobic processes, could you tell me what was your cell yield or your efficiency?

YOUNG: We estimate that for most anaerobic processes, it runs between 1% and 10%. We haven't tried doing cell yield yet. The major problem is separating the organisms from all the materials we have in that system. The lower level of CO_2 may indicate that some of that material is going into cell yield, but we don't have that information.

DiGERONIMO: Based on your stoichiometry, was your balance within 10%?

YOUNG: In the stoichiometry it was in terms of total degradation to CO_2 and methane, so we didn't have a cell yield in that stoichiometry.

DiGERONIMO: Was that one to one, that's my question. The gas formation and disappearance of the product seemed to be one to one. The carbon is accounted for either as CO_2 or methane. It doesn't look as if any of the organisms are using any part of the substrate.

YOUNG: I said, that in our actual measured value, the agreement is not as good, and perhaps that can be accounted for as cell yield.

CHAPMAN: Dr. Young, I'd like to endorse what other people have said this evening about the excellent nature of your presentation. I'm delighted to see certain structural relationships between isovaleric acid and ferulic acid. It seems almost as if the three-carbon side chain of ferulic acid attached to that C-1 position of the ring is the basis for the origin of isovaleric acid. Is it true to say that you only see isovaleric acid when it's derived from ferulic acid, and that you fail to see it when vanillic acid is used as a source of carbon in your experimentation?

YOUNG: We don't have that information. We have used vanillic acid, vanillic enrichments, but we don't have that information as yet.

CHAPMAN: If that is the case then there is clearly a lot of exciting new biochemistry to be discovered in the process. Thank you.

COONEY: I have a question for Steve Orndorff, please. Does your definition of Kepone resistance mean tolerance to 1000 ppm?

ORNDORFF: We initially screened organisms that were resistant at the 100 ppm level. In subsequent testing we found that 100 ppm seemed to be a threshold concentration, in other words, organisms that were resistant at that level would be resistant at nearly any higher levels. Thus we used resistance to 100 ppm as our criterion.

COONEY: So it is 100 ppm or one hundredth of one percent?

ORNDORFF: Yes, but all organisms were found to be resistant to 1000 ppm Kepone.

COONEY: What was the alternate carbon source or sources that you added in order to get degradation or transformation of Kepone?

ORNDORFF: In the pure culture studies we have done, which I did not discuss here, glucose was used. In testing resistance at 1000 ppm, 0.1% yeast extract or proteose peptone was used.

BOURQUIN: I was just reviewing some of our data. When we first read your abstract we were pleasantly surprised because your data were in agreement with our laboratory results. So I was surprised when you said your data conflicted with our results. As I recall, we showed that sensitive organisms showed amylolytic and lipolytic activity which is, I think, what you showed.

ORNDORFF: In the physiological testing, I did not screen the Kepone-sensitive bacteria. The results I presented were only for Kepone-resistant bacteria. The James River isolates primarily were not amylolytic, but in the Baltimore Harbor, approximately 40% of the Kepone-resistant isolates were amylolytic. That was the main point where our data disagreed with your results.

BOURQUIN: If, in our studies, amylolytic organisms were shown to be more sensitive, and you demonstrated fewer resistant amylolytic organisms in a river having been exposed to Kepone, then I think it is reasonable to assume the sensitive organisms may have been eliminated. I interpreted that as in agreement with your data although indirect evidence.

MACALADY: I have a question on Kepone-resistant bacteria. I was really surprised by one number on your slides that you commented on briefly, but I would like to ask another question on it. It seems rather amazing to me that in sediment in the James River you found such a remarkably higher percentage of Kepone-resistant bacteria, in an area where any Kepone was deposited.

ORNDORFF: Yes, there is a problem in hydrology, at the Bermuda 100 station which is about 3 miles upstream. A lot of the Kepone was carried upstream. We don't know how, but there is quite a bit of tidal action in that area. It is essentially fresh water, but it is tidal, with 10-foot tides in that area. Some of the Kepone could have washed back to that area. In addition, there is quite a bit of coliform contamination in that area, and subsequently coliforms are quite resistant to Kepone also. In fact, essentially all of the gram-negative bacteria tested appear to be resistant.

BETTINGER: You said the plasmid related to Kepone resistance also appears to be related to heavy metal and antibiotic resistance. You indicated a rather wide spectrum antibiotic profile, and that you could cure the Kepone resistance but retain its antibiotic, heavy-metal resistance. Is this the same kind of plasmid that Dr. Colwell referred to this morning that could pick up lactose-fermentable ability? I'm curious how you can cure just the Kepone resistance but keep the heavy metal and antibiotic resistance.

ORNDORFF: The Kepone-resistant strain did contain two plasmids. It is quite possible that the other plasmid carried the antibiotic resistance factors, and we cured one of the two plasmids. In subsequent studies we have another isolate which contains only one plasmid and it does demonstrate, in addition to Kepone resistance, resistance to some heavy metals and one or two antibiotics and pesticides. When we cured this strain it also lost its resistance to Kepone. Then, when we tested it for reversion frequency, we found it to be approximately 1.6×10^{-12}. I think that's about the same as for one gene. We don't have confirmatory evidence, but it's possible that one plasmid could mediate some factors which granted resistance to Kepone.

BETTINGER: What was the curing agent?

ORNDORFF: Mitomycin C.

MACALADY: The James River is also famous for another sort of incident that involves chlorination. I'm surprised that you didn't mention anything about testing for residual chlorine levels at the time you were doing other tests. Can you comment on that?

ORNDORFF: Actually, we never thought of doing that. That's a good point. The James River and Baltimore Harbor both receive quite a lot of contamination from many sources. Pesticides as well as industrial effluents in the Richmond and Baltimore areas are contributing to those waters.

JOHNSON: In this Kepone problem, what really is the relevance of using high concentrations of these chemicals? These numbers do not correlate with the amount of Kepone you find in the environment. In other words, we do not detect 100 or 1000 ppm in the sediment.

ORNDORFF: We were trying to exert as much pressure as possible without eliminating the entire population, and are attempting to select for those organisms which are highly resistant, and possibly capable of degradation.

JOHNSON: When you were talking about different physiological groups, their presence or non-presence, what concentrations did you use in making those tests? What types of test were used?

ORNDORFF: I just used the standard procedures for hydrolysis of starch, Tween 80, lipid. The tests were done employing the "core" characteristics of our laboratory and include standard microbiological methods. I did not run those tests with Kepone in the test media.

BAUGHMAN: I have one question I'd like to ask on Kepone, following up on something Dr. Johnson is pursuing. As I recall, the solubility of Kepone at the pH you are working at is about 60 ppb.

ORNDORFF: It's insoluble in water.

BAUGHMAN: How insoluble is that?

ORNDORFF: It is only soluble to about 0.1 ppb.

BAUGHMAN: This brings up the question that concerned us in trying to look at degradation of highly insoluble compounds. These materials are strongly sorbed by microorganisms. Since you cannot get the aqueous phase concentrations to exceed the solubility, I'm wondering how you benefit by increasing the amount of material in that system greatly above the apparent solubility. Do you increase the availability of the materials to the organism? I think it can be shown fairly clearly that you do not.

ORNDORFF: Well, it's true that these chlorinated insecticides, especially Kepone, will be bound up tightly by any organic matter present. They can precipitate out with salts. Some investigators have gotten around, or try to get around the problem by suspending the insecticides in acetone, or sorbing to glass beads, or some other structure which can be suspended in a broth medium. There is probably quite a bit of sorption to the bacterial cell. This cannot be accounted for in our experiemnts. However, we did try to maximize the availability of the compound for the bacteria.

BAUGHMAN; I think perhaps it's a point worth considering by those of us concerned about these studies. I often wonder what we do to the physiology of the organism when we really sock it to them at levels of 1000 or 10,000-fold greater than the aqueous phase.

T. JOHNSON: We're (NMFS) quite interested in the effect of chemicals on fish. Many times microbiologists (and this is one microbiologist who does the same thing) neglect the organisms that we are charged to protect. For example, DDT is considered relatively insoluble in water, yet roughly to 1-5 micrograms per liter (1-5 ppb) is the LC_{50} value for a good percentage of our freshwater game fish. So we should at least consider low concentrations, because they are very important to the fishery resource. When we work at very high concentrations, the relevance of the data to the fishery resources is questionable.

AHEARN: One possible consideration in relation to the effect of water insoluble compounds on microorganisms would be the microcosm effect in an oil or lipid layer. The compound could be available to microorganisms in that microcosm at a rather high concentration.

COLWELL: I feel compelled to speak to the point concerning the concentration of Kepone used in our studies. What is neglected in the discussion is that the effect we are testing is that which alters the growth of the organism in the presence of Kepone. In Steve's work, he used several concentrations, including a 1000 ppm and lower concentrations. In the case of one system employed, when the bacterium was cured of the plasmid, it was no longer capable of growth on the Kepone. Biodegradation aspects of this work should be emphasized. The concern about the concentration of Kepone used is understandable, but in effect this was, in the case of our sediment studies, to negate binding of the Kepone by providing an excess; that is, to have available Kepone in solution.

ORNDORFF: Everyone has put too much emphasis on the 1000 ppm concentration. This was only one test, carried out in a broth culture, merely to see if the organisms remained viable at that concentration. None of the other tests were done using a 1000 ppm concentration of Kepone.

RIBBONS: I wonder if you could tell me the evidence for the plasmid?

ORNDORFF: Yes, it was separated by agarose gel electrophoresis, developed with ethidium bromide and visualized under long-wave UV.

RIBBONS: You showed two plasmids. Did you show the absence of plasmids with the cured strains?

ORNDORFF: Yes, we did.

RIBBONS: And did you transform back into a cured strain?

ORNDORFF: That's very difficult with *Pseudomonas*. We have not been able to do that yet.

RIBBONS: Do I infer, then, that the cured strain had antibiotic resistance and heavy-metal resistance which was not plasmid-borne?

ORNDORFF: In one isolate, yes. But it is possible that this may have been on another plasmid which could have possibly integrated into the cell chromosome.

BOURQUIN: Dr. Cerniglia, you were talking about degradation of aromatic hydrocarbons by fungi. Do you think that fungi actually compete with bacteria to any great extent in the environment?

CERNIGLIA: As far as aromatic hydrocarbons are concerned, I don't think that there is a study being done to look at the fate of these compounds with respect to fungal degradation. I don't think we actually know. That's one of the things I've been trying to emphasize in our task group discussion. Are fungi playing a role in degradation of these types of compounds? We know that they do in pure cultures.

BOURQUIN: What about partial metabolism of some of the polynuclear aromatics? Do you think fungi might partially metabolize compounds and actually form, say, arene oxides or something that could accumulate?

CERNIGLIA: From our study it seems it is very likely.

AHEARN: A number of questions have been voiced during break. I will indicate these questions and perhaps we can have open discussion on these. I think this is directed to Dr. Liu. It has to do with the disappearance of the PCB from the culture system due to absorption rather than metabolism. Perhaps Dr. Liu can discuss this point. The other question was a point of clarification in relation to the Kepone. Whether or not there was a metabolite demonstrated in this study, whether or not there was growth.

ORNDORFF: Since that sediment experiment, we've done pure culture work. We've been able to identify the same metabolite in pure culture broth using glucose as a carbon-energy source, so that it appears as if it is a cometabolic effect. In fact, I even have the GLC tracing of it if you would like to see it. Several pure cultures, including isolates from the James River, Bailey's Creek, and the sewage treatment plant at Hopewell, Virginia, were examined. All of these isolates yielded chromatograms after growth in a Kepone medium that appear basically the same as this one. The major peak that you see to the left of the Kepone is absent in all of the controls.

GARNAS: Can you tell us what kind of confirmational evidence you have that this metabolite is the monohydro-Kepone?

ORNDORFF: There are problems in measuring the derivative. It appears to be a monohydro derivative. The concentrations are extremely low and are barely amenable to gas-liquid chromatographic analysis. Cells have been grown in batch culture and as much of the product as possible has been recovered. Mass spectral analyses of these extracts are in progress.

GARNAS: I am very happy that you came here to give the paper. We've completed a detailed study on the fate, movement, and possible biodegradation of Kepone in various estuarine systems. To date, we haven't seen any evidence of degradation, and we specifically looked for the mono- and dichlorinated products. We used ^{14}C materials to get a very acceptable budget analysis on our system. So we are quite interested in seeing a little more confirmation of this peak.

ORNDORFF: As I said in the talk, Dr. James Smith at Allied Chemical has analyzed samples taken from the Kepone dump at Hopewill. He and his colleagues found monohydro derivatives.

GARNAS: It is speculated that perhaps the compound was in the original synthesis batches that were dumped.

ORNDORFF: No, the synthesis of Kepone is a clean reaction, a very efficient reaction.

GARNAS: I will differ with you strongly on that point.

ORNDORFF: From what I've been told by Allied Chemical, they claim it is a very efficient reaction. They claim the monohydro derivative they've found in the soil samples was not present before dumping.

GARNAS: They did find some of the dechlorinated products in some aquatic species also. But where you find residues, it doesn't necessarily mean that they were produced in that site. It is speculated, and there is good evidence to back it up, that the discharging of Kepone was not in a pure state. It was distributed with other materials including hexachlorocyclopentadiene and the residual from batch synthesis. The compound is not synthesized pure. There is a certain amount of impurity in it. It is very difficult to obtain 99.9% pure analysis standards to work with. So I submit that the materials that were probably found in those sewage treatment sediments could have been introduced in the original disposal of material.

ORNDORFF: That may be true. Nonetheless, in our pure culture studies, there was no monohydro derivative in the starting material.

GARNAS: I agree with you that the gas chromatographic analyses are quite difficult. I think it is very important to keep in mind that in electron capture GC, cleanup conditions become very critical and proper controls become very critical. Peaks are due to the electron-capturing nature of the molecule; and unless you have a good standard available, peak size is no indication of the level of conversion, if it is happening. I still submit that it's a very critical issue, and I think it requires substantiation.

BOURQUIN: I have a question concerning the pure culture studies. Have you ever looked at another chlorinated compound to see if there is dechlorination other than with Kepone? To see if there is a non-specific reaction for a cage structure or something like, for example, can you show dechlorination of mirex?

ORNDORFF: No, we haven't, but we are looking at the precursors used in the Kepone synthesis--hexachlorocyclopentadiene and dicyclopentadiene.

BOURQUIN: I don't know if I misunderstood you, but I thought you said your product was 1/10 of 1% of what was added?

ORNDORFF: That's correct.

BOURQUIN: That seems extremely small.

DiGERONIMO: A question for Dr. Pritchard or Dr. Bourquin about Kepone. Do you think not seeing Kepone degradation in your systems could be accounted for because of the enrichment period in James River sediments could cause organisms to evolve or mutate and they have been selected, whereas you people are working with a rather fresh sample?

BOURQUIN: We are using James River sediment in our systems.

DiGERONIMO: That answered my question. I have to bring it up because from our work in the sewage system, we found that responsible organisms were in very low numbers. They probably could be missed depending on your sample size. This may be a reflection of the complexity of one microcosm showing something vs another microcosm.

BOURQUIN: It could be we have missed or not selected for a specific organism. I think the whole question is very interesting. However, I'm wondering about the 0.1%. What does it mean in terms of degradation? Is it significant in the environment, if it is a product? Something we have found in our studies with Kepone, but haven't reported yet, is another product in our systems. It amounts to 10-20% of the Kepone. Other people at Fish Pesticides Laboratory, Dave Stalling and his group, have reported a similar product in fish of up to 40 or 50% of the Kepone added. These are not dechlorinated products. They are less polar compounds and they are probably conjugated with lipoprotein. This is not confirmed, because mass spectral analyses have not been performed.

DiGERONIMO: You commented on a 0.1% product. Just to plug cometabolism, actually 0.1% of the compound for a cometabolic system would probably be a predictable amount based on cell mass. Because you are not expecting any increase in cell mass; therefore, from our experience, you wouldn't expect a total turnover or any appreciable amount of turnover of a compound. Secondly, I have to make one more comment about the contamination of compounds. When we first started out, we were using 2-3-6 trichlorobenzoic acid. We were finding really fantastic results until we purified it. Then we found these results disappeared, even though we were assured 99% purity; it wasn't. So a lot of the data we did accumulate on the 2-3-6 and a lot of the previous data became suspect because of the purity of the compound.

BOURQUIN: Just one other thing on looking for Kepone metabolites. In all systems used to study Kepone at our laboratory, there have been GC searches as well as radioisotope searches for metabolites. In the toxicology laboratory there have been any number of studies using flowing water bioassays, with fish, crustaceans and oysters. All of these samples were checked for monohydro- and dihydro-Kepone as well as any other product. They never really found any products. In our systems we've run continuous-flow degradation systems and a number of the eco-core experiments in which we have used fresh estuarine sediments and water from the local Range Point salt marsh previously uncontaminated with any type of pesticides that we know of, and using James River sediments. We have looked for degradation in systems under anaerobic conditions (purged with nitrogen), and in systems with additional growth substrates like naphthalene or glucose added to increase microbial activities or to increase aromatic hydrocarbon-degrading activity within the sediment or water system. We've looked at flowing water systems using 10-gallon aquaria where ^{14}C-Kepone was added to either James River or Range Point sediments (two different systems). Here again we did find the conjugate product in association with decomposing lug worms. In all cases where we find this other product, it has always been in anaerobic conditions, but in no case have we found any other type of metabolite.

COLWELL: Have you ever done any pure culture studies?

BOURQUIN: Yes, we have. We've got a Corynebacterium-like organism that decomposes camphor as a sole carbon source. We were looking at this organism as a possible source for a fortuitous dechlorination reaction. We don't have any real positive evidence for that either.

COLWELL: Have you studied in pure culture any organism that exhibited any Kepone resistance?

BOURQUIN: Yes, meaning the organism grows on Kepone agar plates; there are any number of those. We didn't do any culture studies that would show anything like metabolism of Kepone.

GIBSON: I'm surprised to find myself with an unexpected interest in Kepone. First of all, I would like to ask if there any idea of the mechanism of the toxicity of this compound.

ORNDORFF: In studies using Tween-80, we found that in the presence of 10 ppm that Kepone was toxic to the cells. So there may be some sort of permeability factor that enables the organisms to be resistant to Kepone. Since these are very lipophilic compounds they may be bound up in the LPS of the cells, we don't know. We can only guess at that.

GIBSON: Just looking at that structure, you would predict that no organism in its right mind would look at it. If you look at it chemically, as Peter Chapman pointed out, it is a chloromethyl ketone which will react with sulfhydryl groups, and will probably be a very toxic compound. I was wondering, when an organism becomes resistant, do you think it's due to biotransformation?

ORNDORFF: It could be. Your guess is as good as mine. I don't know.

GIBSON: You said the two things went hand in hand, that if it lost the ability to transform, it also becomes Kepone sensitive?

ORNDORFF: In the isolates tested in the plasmid screening, those isolates that were cured were no longer resistant to Kepone. But we are still in the process of testing whether these cured isolates have any ability to transform the molecule. I suspect that they will not be able to.

GIBSON: I was a little worried about that number of reversion, one in 10^{-12}, did you say?

ORNDORFF: 1.6×10^{-12}, approximately the same as for one gene in one isolate.

GIBSON: Did you say you couldn't transmit the plasmid back?

ORNDORFF: We haven't done the experiments yet but we are working on it. It's very difficult with pseudomonads, for obvious reasons. They are not like the *Escherichia coli* system.

GIBSON: I guess I don't understand how you got the number, then.

ORNDORFF: This was the reversion frequency of the resistant organism, spontaneous reversion frequency.

GIBSON: For George Baughman or Al Bourquin, when you are looking at a really insoluble substance like this, how can you have Kepone plates at levels that are way above the solubility level and be able to say certain concentrations are toxic?

ORNDORFF: This is a real problem. Testing resistance on agar plates is really a very non-specific test and doesn't reveal much information. We screened all isolates on a 100 ppm Kepone agar, then picked each individual colony and retested it in broth culture, which is a better method for testing for resistance to Kepone than agar, for obvious reasons.

GIBSON: So you still don't know what your concentrations are?

ORNDORFF: That's true. Kepone usually precipitates out if you've got high salts in a broth, or it will bind up with any of the other organic matter, whatever may be in the broth.

BOURQUIN: In addition to agar plate toxicity testing, we are confirming our results using O_2 uptake studies on the oxygraph.

GIBSON: If it's taken this long just to get rid of one chlorine and there's a heck of a lot of chlorine still on that molecule, are you ready to predict how long that molecule is going to stay in the James River?

ORNDORFF: No.

BETTINGER: Just a comment concerning agar plates and insoluble materials. I don't know if the analogy can be drawn, but in the *Salmonella*-microsome assay, many times we look at compounds which precipitate but still show dose response. We're kind of at a loss to explain that. I don't know if that's really relevant, it's just an observation.

GIBSON: That really worries me, because in the Ames test for a compound to exert its effect, it has to be electrophilic. The most reactive compounds that are known are very, very unstable and react with any nucleophilic site they contact. So if you put a compound in that precipitates, plus all the hazards they have to go through before it exerts its mutagenic effect, that's the compound that scares the heck out of me.

BOURQUIN: Dave, in answer to your question on high concentrations, in our toxicity tests, we were looking at the sensitivity of organisms to Kepone at concentrations of .02, 0.2, and 2 ppm. These are suspensions, and you are right, you really don't know the concentration. It's not like an antibiotic test where you have diffusion through the agar. What we are showing in those tests is a positive or negative result. From there, any confirmation on toxicity is backed up by oxygen uptake study, where a low concentration of Kepone is added in solvent, to a whole cell suspension and additional substrate. What we've done from there then is to back calculate to get a proportionate ratio of Kepone concentration to a measured number of bacteria from an environmental sample.

GIBSON: I realize that these experiments are very difficult; we work with insoluble substrates, too. They are a real pain. Is it possible to take ^{14}C-Kepone and just add it to the bacteria and find out how much is adsorbed to the cells? Does that actually happen?

BOURQUIN: Sure. You mean in pure culture?

GIBSON: Yes.

BOURQUIN: It could be done very easily. We normally add it in acetone to the medium, but it is rapidly adsorbed to the cells. In fact, I think it is 90% or more of the radioactivity adsorbed in the cells almost immediately in these high density experiments.

BAUGHMAN: I'm not sure what relevance it has to the metabolite question, but you might bear in mind something that I think is often overlooked in much environmental work. First, there are very few organic compounds you can obtain more pure than probably 99.5%; 99% would be very good; and you can almost always expect impurity levels on the order of the 0.1% or better. Now, clearly, if you have a compound with a solubility of 0.1 ppm and if you add 100 ppm of that material to a system and if that material you add has 0.1% of impurity, you have an equal amount of impurities potentially in solution to what you have in the material you are testing. And we've seen examples where this can play all manner of hazard with the kinds of measurements that are made in environmental work. For example, in the measurement of octanol/water partition coefficients. There is a tremendous motivation to work with labelled materials for environmental studies of any kind. Labelled materials are particularly noted for being impure, and since you are looking often at very hydrophobic materials, the impurities are likely to be more polar so you can get orders of magnitude error from that sort of thing.

RAYMOND: Let's switch gears a little bit here, Carl, in the metabolism of aromatics by fungi. Do these compounds conjugate readily like they do in mammalian systems? Or do the intermediates that you see?

CERNIGLIA: In mammalian systems, naphthalene metabolism has been shown by Jerina. Conjugated metabolites have been reported. I have presented evidence looking at soluble organic metabolites, but, yes indeed, the aqueous fractions do contain conjugates.

RAYMOND: And so that in contrast to microbial or bacterial systems where you, I think, probably see less conjugation because of the type of materials, you would not expect it really to be competitive then, would you? At least as far as total degradation?

CERNIGLIA: Yes, I would not.

IVANOVICI: My question is addressed to Dr. Maccubbin. Did you check the microbial populations for viability and if so, how did you do it? I don't recall what you said. I was wondering what sort of viability test you used.

MACCUBBIN: The only reference I made to viability was the viable count obtained by enumerating bacteria on a growth medium and assuming that growth represents viability.

IVANOVICI: The other question relates to your measurements of ATP. ATP is used by some people to determine biomass. Is that why you measured ATP or did you measure ATP concentrations to get an idea of some other aspect of the population?

MACCUBBIN: It was measured in the reference of biomass, not stress.

IVANOVICI: Are you going to be doing any more work in this area?

MACCUBBIN: Not with ATP.

AHEARN: We still have the question that had been posed earlier about the possibility of absorption of the PCBs in the culture system accounting, perhaps, for part of its disappearance. Do we have any comments in relation to that area to the person who posed that question or to Dr. Liu?

SAEGER: The mono- and dichlorobiphenyls are quite readily degradable and Aroclor® 1221 is basically mono- and dichlorobiphenyls.

GIBSON: We find exactly the same and that work has been published in the literature. Although some of the trace amounts of the higher chlorinated biphenyls that are in 1221 still stay behind. We haven't been able to demonstrate any degradation of those.

DEGRADATION METHODOLOGY

Chairperson, R. L. Raymond

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DEGRADATION METHODOLOGY: CHEMICAL-PHYSICAL EFFECTS

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ABSTRACT

This review of the physical and chemical factors that affect the distribution and degradation of pesticides in aquatic environments treats processes such as volatilization, partitioning, hydrolysis, oxidation, reduction, and photochemical reactions. Reactions in sterilized and nonsterilized soils are discussed and compared.

At a symposium on "Nonbiological Transport and Transformation of Pollutants on Land and Water" held at the National Bureau of Standards in 1976, in a paper entitled "Oxidation and Reduction of Pesticides in Soils and Sediments," I discussed some of the difficulties in drawing a clear distinction between reactions that are solely chemical in origin and reactions that accompany biological processes. The proceedings (29) provide a useful starting point for a discussion of physical and chemical factors that affect distribution and degradation of pollutants.

PHYSICAL FACTORS

Without microbial or chemical degradation, the concentration of a pollutant in an aquatic system will be reduced by physical factors, like dilution, adsorption, or volatilization. These processes in turn will be affected by the physical properties of the molecule, particularly water solubility, vapor pressure, and polarity. Insoluble or adsorbed pollutants will be transported or deposited. Dissolved materials also are subject to transport, adsorption and subsequent deposition. Substantial effort has been devoted to the study and modeling of such processes. A model developed by Crawford and Bailey (4) to describe movement of pesticides and nutrients in agricultural lands took into account factors that are important in nonpoint pollution: movement of pollutants in runoff over land with subsequent percolation of dissolved materials to ground water aquifers, the adsorption of pollutants on soil particles followed by erosion and movement of the soil as sediment, and the erosion of pollutant particles (4).

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Adsorption and desorption are important because they affect the chemical reactivity and biological availability of pollutants. There has been much recent discussion on the fate of bound residues and the question of their further significance as pollutants (14).

The loss of pollutants by volatilization from water bodies was studied by Mackay and collaborators (19). If the pollutant concentration in water and in the atmosphere and Henry's law data are known, approximate values for the mass transfer coefficients, K_L (the liquid phase mass transfer coefficient) and K_g (the vapor phase mass transfer coefficient) can be calculated. A mass flux rate can then be calculated from these values to within a factor of about five. This information may be sufficient to predict whether volatilization represents a major pathway of loss for a given pollutant. However, several complications limit the value of this treatment as a tool for prediction of environmental fate. The surface layer of organic matter normally present on natural water bodies, the process of spray formation and transfer by bursting bubbles, and the presence of pollutants as micelles or colloids, rather than as true solutes, are factors that must be considered in a treatment of volatilization; however, their significance is not fully understood.

An important factor in dispersal and transformation of organic pollutants is their partition between lipid and aqueous media. The partition coefficient is important in constructing predictive models, as well as in considering the availability of a pollutant as a potential microbial substrate. Accumulation by biota is extremely dependent on the lipid-water partition coefficients may be significant in relation to the transport and fate of pollutants in the biosphere. In the same context, the potential changes in solubility that result from association of pesticides with surfactants and other formulation ingredients may affect their reactivity.

Finally, the availability of pollutants may be decreased by precipitation from solution. This mechanism may be important in removing metals from fresh water or marine environments.

CHEMICAL FACTORS

Superimposed on the physical processes that change the concentration of a pollutant in water are chemical reactions that can transform it by various mechanisms. A major difficulty in assessing the importance of chemical transformation is the paucity of information concerning reactions in the many hererogeneous systems involved. Experimental reaction rates are usually measured at precisely controlled temperature and pH in homogeneous solution, whereas in the environment, many pollutants are adsorbed on the surface of particulate matter. Natural conditions probably favor the concentration of organic pollutants at interfaces, i.e., air-water, air-soil, water-soil. Adsorption and desorption by soil or inorganic media will profoundly affect the rates of chemical or microbial transformation in aquatic systems.

Rates and mechanisms of chemical reactions also are affected by temperature, hydrogen ion concentration (pH) of the medium, redox potential, and ionic concentration. These factors probably will be relatively constant within large bodies of water, but not necessarily at interfaces. Newly deposited sediments, rich in organic material, provide a reducing environment, but the upper sediment layers and the water column above it generally contain an oxidizing zone. At the water surface, sunlight will cause photochemical change. The quantitative significance of photochemical reactions depends on light intensity, a factor that is influenced by many environmental variables.

Biological and chemical transformations take place in a chemical milieu that is defined by parameters such as pH, redox potential and temperature. The growth or decline of specific microbial populations will be influenced by the availability of nutrients and oxygen. As changes in the medium result from microbial processes that may also deplete oxygen or produce acidic metabolites, there will be accompanying changes in rates and products of purely chemical reactions.

Patrick and his coworkers (21) have discussed the physicochemical factors that regulate solubility and bioavailability of heavy metal pollutants. Their discussion is concerned with the chemical state of the heavy metals, which in turn is regulated by microbial activity occurring within the system. The potential toxicity of sediments that contain metals depends on the concentration and chemical form of the toxic metals as well as on the physicochemical and biological conditions of the sediment. Sediments are generally anaerobic, and the bulk sediment solids with which most of the toxic metals are associated will remain anaerobic when subaqueous disposal methods are used for dredged sediments. However, major changes in mobility and biological availability may occur if the oxidation-reduction conditions at a disposal site differ from the physicochemical environment of the sediments at the dredging site. Such changes may occur when an anaerobic sediment is deposited on land where oxygen becomes freely available or if an aerobic sediment is dredged and becomes mixed with an anaerobic sediment-water system.

Under aerobic conditions, sulfide can be oxidized to elemental sulfur or sulfate. Other transformations include oxidation of ferrous to ferric ion, manganous to manganic ion, and organic nitrogen to nitrite or nitrate. These reactions produce increases in acidity with concomitant changes in stability and bioavailability of metal ions. The amount of available iron may be changed by as much as three orders of magnitude. Thermodynamic equilibria may be useful for predicting the forms of iron, zinc, cadmium, mercury, or lead. Metals may be present as free soluble cations, soluble organic or inorganic complexes, easily exchangeable cations attached to clays, precipitated metal hydroxides, precipitates with iron or manganese oxyhydroxides, insoluble organic complexes, insoluble sulfides, or residual forms. However, the diversity of the components of natural aqueous systems and the variability of their concentrations limits the predictive value of thermodynamic equilibria. In natural systems, true chemical or biological equilibria are rarely, if ever, likely to exist. Redox potential-pH diagrams may provide useful indications of variations in metal speciation that result from changes in physicochemical environment.

Oxidative processes will alter humic acids and organic matter present in the sediment; thus, the process of complex formation will be affected.

The major factors influencing equilibria are redox potential and pH. Under aerobic conditions, the former may vary from 300 to 600 mv (strongly oxidizing), and surface waters containing dissolved oxygen usually range in pH from 5.0 to 8.5 (moderately acid to mildly alkaline). Anaerobic sediments are generally almost at neutral pH (6.5 to 7.5), and they are moderately to strongly reducing (100 to 400 mv or less).

The latter potentials are sufficient to reduce several types of organic molecules. Toxaphene undergoes extensive alteration in flooded soils to yield products that appear to be formed by reductive dechlorination (22). DDD [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane] is formed from DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] under anaerobic conditions in flooded soils or sediments (7). From the measured reduction potentials required for the electrolytic reduction of the dinitroanilines to *m*-phenylenediamines via the phenylhydroxylamines, a nonbiological pathway for degradation of dinitroanilines is feasible under anaerobic flooded conditions (27). In summary, such reactions may not be strictly metabolic but chemical transformations will be mediated by factors that depend on microbial processes occurring in the system.

Further examples of associated chemical reactions are the purely thermal reactions of reactive metabolites. The incubation of chloroanilines with *Fusarium oxysporum* gives chlorophenylhydroxylamines as initial products. Phenylhydroxylamines are very reactive and rapidly condense to produce azoxybenzenes. The latter are frequently obtained as products or by-products during the laboratory preparation of the phenylhydroxylamines (13). Thus, the presence of transformation products obtained by incubation of a xenobiotic chemical with a microbial system may not always provide direct evidence concerning metabolic pathways or enzymic processes, unless we examine the evidence critically.

HYDROLYSIS

Water is a readily available reactant in aquatic systems. However, many pesticides and pollutants are relatively stable toward hydrolysis at neutral pH and environmental temperatures. The presence of soil may considerably alter hydrolysis rates, depending on soil type, pH, and salt concentration. Simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine], atrazine [2-chloro-4,6-bis(isopropylamino)-*s*-triazine] and propazine [2-chloro-4,6-bis(isopropylamino)-*s*-triazine] rapidly undergo replacement of chlorine to give the corresponding 2-hydroxy compounds with maintained at 30°C in various soils (9). The criteria for establishing that such reactions are chemically, and not biologically, mediated will be discussed later, but the rate of hydrolysis of the triazines was not affected when the soils were sterilized with sodium azide.

A few pesticides are hydrolyzed in distilled water. Heptachlor (1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene) gives 1-hydroxychlordene in distilled water at room temperature (1), and the soil fumigant, 1,3-dichloropropane, is hydrolyzed to 3-chlorallyl alcohol at pH 6.9-7.5 (3); this hydrolysis is catalyzed by soil.

The rates of hydrolysis of the organophosphates are very dependent on pH of the medium and, in many instances, the rate increases by a factor of 10 with unit increase in pH. The rate of hydrolysis also is increased by the catalytic effect of soil. Diazinon [*O*,*O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl)phosphorothioate] has a half life of 185 days in water at pH 7.4

and 20°C, but in cupric chloride solution and in copper-montmorillonite clay suspensions, it is quantitatively hydrolyzed within 24 h (20). Parathion (*O,O*-diethyl *O*-*p*-nitrophenylphosphorothioate) and malathion [*S*-1,2-di(ethoxycarbonyl)ethyl *O,O*-dimethylphosphorothioate] also are hydrolyzed more rapidly in soils than in aqueous solution.

Other pesticides, known to undergo hydrolysis, include the esters of phenoxyacids and the carbamates. The esters of 2,4-D[2,4-dichlorophenoxy)acetic acid] and other carboxylic acids are rapidly hydrolyzed in soil.

OXIDATION

Oxidation may be responsible for degrading a large fraction of the pollutants present in aquatic systems and soils. Oxidative processes may result from microbial action, photo-oxidation, the direct attack of oxygen, or an activated oxidizing species. Variables affecting these factors include pH, soil type, metal ions, photosensitizers, oxygen content of the sediment, temperature, and sunlight. Microbial systems are responsible for many oxidative transformations but the distinction between biological and nonbiological reactions is not an easy one. Several complex catalytic systems involving metals can provide models for oxidizing systems. The products resemble those from biological oxidation and there are close analogies between them in that they both involve generation of activated species of oxygen.

The role of transition metals as oxidants in aqueous systems requires further investigation. In the presence of complex organic molecules, metal ions may take part in oxidation processes that are closely analogous to biological oxidations mediated by enzymes.

Several chemical systems mimic the reactions of microsomal oxidases responsible for in vivo oxidation of aromatic compounds and other organic compounds foreign to the organism. These oxidases include drug hydroxylases, demethylases, and *N*-oxidases, and they require TPNH and molecular oxygen together with a metal. Chemical model systems have provided some clues as to the nature of activated oxygen species in enzymic reactions. Fenton's reagent (iron salts, hydrogen peroxide, and sulfuric acid) is a source of hydroxyl radicals and will hydroxylate aromatic systems or dealkylate amines or ethers. Other model oxidation systems have been described, like that of Udenfriend et al. (28) containing ferrous sulfate, ascorbic acid, and ethylenediaminetetraacetic acid in a buffer. Hamilton et al. (8) have studied hydroxylation of aromatic compounds in the presence of ferric iron together with hydrogen peroxide and catechol, but the reactive species was probably not the hydroxyl radical. Hydroperoxy or peroxy radicals seemed unlikely because they did not react very well with aromatic systems. The formation of an iron-catechol complex responsible for direct transfer of oxygen to the substrate was postulated. This model system bears some analogy to the iron-porphyrin enzymes. Views of the biological mechanism of oxygen activation involve analogous mechanisms in which cytochrome P-450 forms ferric hydroperoxide-type complexes or ferric superoxide-type complexes with a substrate.

Few examples of nonbiological pesticide oxidation can be substantiated. Aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,4,8,8a-hexahydro-1,4-*endo*, *exo*-5,8-

dimethanonaphthalene) is slowly converted to dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*, *exo*-dimethanonaphthalene) in soils (18), and this reaction is considered to be nonbiological. However, documented instances of environmental nonbiological oxidation, not mediated by photochemical action, are rare.

REDUCTIVE TRANSFORMATIONS

"Reduction" of organic compounds usually implies reactions like the saturation or addition of hydrogen to carbon-carbon, carbon-oxygen, or nitrogen-oxygen multiple bonds. Replacement of halogen by hydrogen is important in many pesticide pollutants. In the laboratory, these reactions require vigorous chemical reducing agents or hydrogenation using a metal catalyst. Such conditions are not characteristic of the environment, in which reducing reactions must occur under mild conditions, e.g., disproportionation reactions with a low activation energy threshold, or reactions brought about by complex electron-transfer systems. Models for the latter may be constructed if we can determine the type and role of biological catalysts of related function.

Metabolism of toxaphene by rat liver microsomes requires NADPH and anaerobic conditions. Khalifa et al. (16) suggested that reduced cyctochrome P-450 acts as the reducing agent under these conditions. They found that reduced hematin reacted with toxaphene to cleave C-Cl bonds and pointed to the value of such iron (II) protoporphyrin systems as models for understanding the degradation of some chlorinated hydrocarbons. Mirex (dodecachlorooctahydro-1,3,4-metheno-1*H*-cyclobuta[*cd*]pentalene) also reacted with reduced hematin to give reductive dechlorination products (10).

The reduction of several pesticides under strongly anaerobic conditions in flooded soils has been mentioned earlier in this discussion and it seems likely that purely chemical mechanisms are involved.

PHOTOCHEMICAL PROCESSES

Photochemical processes depend on the presence of light as a reactant. Wavelengths in the 280 to 320 nm region (UV-B region) of the spectrum contain sufficient energy to bring about chemical change by the rupture of a covalent bond. Sunlight contains radiant energy in the 300 nm region, although the actual wavelengths and intensity that reach the earth's surface are dependent upon many environmental factors, in addition to latitude, elevation, and time. In an aquatic medium, light energy becomes attenuated with depth, depending upon wavelength and the absorption coefficient of the medium.

The simplest photochemical processes are "direct" processes involving the breaking of a single bond with the formation of two radicals (homolysis). Products are formed by the subsequent reactions of these free radicals. A common free radical reaction is the abstraction of hydrogen from a suitable donor (photoreduction) (22). Reaction with an oxygen molecule is also a common reaction of free radicals. Oxidation of olefinic compounds in the environment proceeds by this route.

Alternatively, the rate of nucleophilic substitution may be enhanced by light (photonucleophilic reactions) (5). The hydrolysis of chloracetic acid involving attack by the hydroxyl radical is much more rapid in the light than in the dark (30).

Rearrangement of bonds within the molecule can occur: the formation of new ring systems or other isomerization reactions commonly are encountered, as in phytolysis of heptachlor (25).

Indirect or sensitized reactions involve an intermediary or sensitizer molecule that absorbs light and, by energy transfer to a second molecular species, brings about a chemical change in the latter. Thus, photochemical alteration may be induced in a molecule that does not absorb sunlight, if sensitizers are present in the solution. Sensitizers are common in natural waters, although their chemical nature is speculative. The rate of photolysis of a pollutant may be more rapid in river water that appears dark brown than in "pure" water.

Although direct oxidation of molecules by reaction with oxygen is rare, oxidation by activated species containing oxygen is an important biological process, and the photochemical generation of activated oxygen containing species may be important in the environment.

"Dye-sensitized photooxidation" refers to the oxidation of a species by singlet molecular oxygen generated by irradiation of a dye solution through which oxygen is diffused (15). Other photochemically generated, activated oxidizing species may include the radicals $RO\cdot$, $RO_2\cdot$ and $HO\cdot$.

REACTIONS IN STERILIZED VERSUS UNSTERILIZED SOILS

In some instances of pesticide degradation in soil, it has proved very difficult to establish whether the reactions observed were biological or chemical because it was impossible to isolate individual microbial species that would bring about the specific reactions. An example is the degradation of amitrole (3-amino-s-triazole) to CO_2 in nonsterile soils. Amitrole was also degraded in azide- or ethylene oxide-sterilized soils but not in those sterilized by autoclaving. The addition of mixed cultures of microorganisms, isolated from soil in which amitrole had been rapidly degraded, to autoclaved soil caused only slight degradation. Organic amendments added to soil increased microbial activity but reduced amitrole degradation (12). The addition of iron and other metallic salts increased the degradation rate. From these data, it was concluded that chemical degradative routes predominated in the three soils examined. Several free-radical generating systems cause rapid decomposition of amitrole, and the degradation processes affecting amitrole in soils may be closely analogous to those mediated by free radicals, like the hydroxyl radical or photochemically-generated free radicals. Maleic hydrazide is degraded in a similar manner (D. D. Kaufman and N. A. Kaloyanova, private communication).

Sterilization by autoclaving may affect not only the microbial population, but also the chemical and physical structure of soil. Thus, nonbiological processes may be precluded as a result of such treatment. The use of microbial inhibitors, fumigants, irradiation, or other methods of reducing microbial activity is equally open to criticism, because these techniques often result in altered soil reactivity or the presence of reactive residues. For example, we identified a reaction product of chloroaniline and ethylene oxide after incubating the former in ethylene-oxide sterilized soil (J. R. Plimmer and U. I. Klingebiel, unpublished).

Some of the effects of sterilizing soil with propylene oxide, sodium azide, or by autoclaving were investigated by Skipper and Westermann (26). The chemical and biological properties of the soil were altered as indicated by their effects on pH, and germination and growth of wheat and alfalfa.

Fletcher and Kaufman (6) studied the effect of sterilization on the degradation of 3-chloroaniline in soil. They found that the role of peroxidase enzymes was very important but that residual peroxidase activity depended upon the mode of sterilization. Azide treatment or autoclaving inhibited soil peroxidase 100%, whereas ethylene or propylene oxide inhibited soil peroxidase only 4.8 and 8.6%, respectively.

Investigation of ethylene thiourea degradation revealed that the initial degradative step, conversion to ethylene urea, occurred chemically in autoclaved soil. Subsequent degradation was microbial in origin (11).

IMPLICATIONS

The study of the fate of 2,4-D esters in surface waters, by Zepp and his colleagues at the EPA Laboratory in Athens, Georgia (31), is almost unique in its attempt to compare the competing results of evaporative, hydrolytic, and photochemical processes (Table 1). They examined several 2,4-D esters in basic waters, and hydrolysis was the most important process for the methyl, 1-butyl, 1-octyl and 2-butoxyethyl esters. In acid water, the relative significance of hydrolysis depended upon the ester structure. Overall loss was more rapid in acidic than in basic waters. Photolysis was most important for the butoxyethyl ester. Vaporization was most important for the octyl and butyl ester, and both vaporization and photolysis were important processes for the methyl ester. Chemical and photochemical processes transformed the butoxyethyl ester more rapidly than the other esters. The product of hydrolysis, 2,4-D, is insoluble and nonvolatile. It is removed from natural waters by photolysis and the half life was calculated as 20 days.

TABLE 1. 2,4-D ESTERS: COMPARISON OF 25°C HYDROLYSIS, PHOTOLYSIS AND VAPORIZATION HALF LIVES IN DAYS (31)

Ester	Hydrolysis		Direct photolysis	Vaporization
	pH 9	pH 6		
Methyl	0.04	44	29	21.7
1-Butyl	0.2	220	--	1.1
1-Octyl	0.2	220	--	11.5
2-Butoxyethyl	0.02	26	16	895

The vaporization calculations were based on the treatment used by Mackay (19) and photolysis half-lives were obtained by a computer program which gives the integrated sunlight intensity at various depths in water. When photolysis was conducted in Suwannee River water the rate was twice that in distilled water.

Laboratory data can be used to predict rates of dissipation of environmental pollutants by physical and chemical pathways (31). These data were obtained by precise well-established techniques, using purified compounds. An important question that arises is that of the quality and quantity of data needed to provide adequate output from models. How sensitive is the model to the input and what degree of precision is necessary in obtaining such data?

The prediction of rates of environmental degradation is a problem of some consequence. Model microcosms can be used to obtain rates of dissipation under simulated environmental conditions and they are useful in identifying microbial and physicochemical products and processes. However, their predictive value must be tested in terms of real-world phenomena. Patrick et al. (21) have indicated that toxic metal contamination of wastes can be managed somewhat by controlling the properties of disposal or dispersal sites. From relevant data on soils and waters, it should be possible to construct models that provide some indication of the impact of pollutants and their potential residence time in a specific situation.

LITERATURE CITED

1. Bowman, M. C., F. Acree, Jr., C. S. Lofgren, and M. Beroza. 1964. Chlorinated insecticides: fate in aqueous suspensions containing mosquito larvae. Science 146:1480-1481.

2. Branson, D. R. 1975. Predicting a bioconcentration potential of organic chemicals in fish from partition coefficients, p. 99-118. *In* G. D. Veith and D. E. Konasewich (eds.), Proc. Symp. Structure-Activity Correlation in Studies of Toxicity and Bioconcentrations with Aquatic Organisms.

3. Castro, C. E., and N. O. Belser. 1966. Hydrolysis of *cis*- and *trans*-1, 3-dichloropropene in wet soil. J. Agric. Food Chem. 14:69-70.

4. Crawford, N. H., and G. Bailey. 1976. Hydrologic transport processes for pollutants in water and on sediments. *In* Symposium on Nonbiological Transport and Transformation of Pollutants on Land and Water: Processes and Critical Data Required for Predictive Description. National Bureau of Standards, Gaithersburg, Maryland. Program and Abstracts, unnumbered.

5. Crosby, D. G., K. W. Moilanen, M. Nakagawa, and A. S. Wong. 1972. Photonucleophilic reactions of pesticides, p. 423-433. *In* Proc. Symp. Environ. Toxicol. Pestic., U.S.-Japan.

6. Fletcher, C. L., and D. D. Kaufman. 1974. Effect of sterilization methods on 3-chloroaniline behavior in soil. Abstracts, Am. Chem. Soc. 168th National Meeting, Atlantic City, N.J.

7. Guenzi, W. D., and W. E. Beard. 1967. Anaerobic biodegradation of DDT to DDD in soil. Science 156:1116-1117.

8. Hamilton, G. A., J. W. Hanifin, Jr., and J. P. Friedman. 1966. The hydroxylation of aromatic compounds by hydrogen peroxide in the presence of catalytic amounts of ferric ion and catechol. Product studies, mechanism, and relation to some enzymic reactions. J. Am. Chem. Soc. 88: 5269-5272.

9. Harris, C. I. 1967. Fate of 2-chloro-*s*-triazine herbicides in soil. J. Agric. Food Chem. 15:157-162.

10. Holmstead, R. L. 1976. Studies of the degradation of mirex with an iron (II) prophyrin model. J. Agric. Food Chem. 24:620-624.

11. Kaufman, D. D., and C. L. Fletcher. 1973. Degradation of ethylenethiourea in soil. Abstracts, Am. Chem. Soc. 165th Nat. Mtg., Dallas, Texas.

12. Kaufman, D. D., J. R. Plimmer, P. C. Kearney, J. Blake, and F. S. Guardia. 1968. Chemical versus microbial degradation of amitrole in soil. Weeds 16:266-272.

13. Kaufman, D. D., J. R. Plimmer, and U. I. Klingebiel. 1973. Microbial oxidation of 4-chloroaniline. J. Agric. Food Chem. 21:127-132.

14. Kaufman, D. D., G. G. Still, G. D. Paulson, and S. K. Bandal, eds. 1976. Bound and conjugated pesticide residues. ACS Symposium Series No. 29. American Chemical Society, Washington, D.C. 396 pp.

15. Kearns, D. R. 1971. Physical and chemical reactions of singlet molecular oxygen. Chem. Rev. 71:395-425.

16. Khalifa, S., R. L. Holmstead, and J. E. Casida. 1976. Toxaphene degradation by iron (II) protoporphyrin systems. J. Agric. Food Chem. 24: 277-282.

17. Leo, A. J. 1976. Prediction of solubilization of hydrophobic organic compounds in water. *In* Symposium on Nonbiological Transport and Transformation of Pollutants on Land and Water: Processes and Critical Data Required for Predictive Description. National Bureau of Standards, Gaithersburg, Maryland. Program and Abstracts, unnumbered.

18. Lichtenstein, E. P., and K. R. Schultz. 1960. Epoxidation of aldrin and heptachlor in soils as influenced by autoclaving, moisture and soil types. J. Econ. Entomol. 53:192-197.

19. Mackay, D., and A. W. Wolkoff. 1973. Rate of evaporation of low-solubility contaminants from water bodies to atmosphere. Environ. Sci. Technol. 7:611-614.

20. Mortland, M. M., and K. V. Raman. 1967. Catalytic hydrolysis of some organic phosphate pesticides by copper. J. Agric. Food Chem. 15: 163-167.

21. Patrick, W. H., Jr., R. P. Gambrell, and R. A. Khalid. 1977. Physicochemical factors regulating solubility and bioavailability of toxic heavy metals in contaminated dredged sediment. J. Environ. Sci. Health A12:475-492.

22. Plimmer, J. R. 1971. The photochemistry of halogenated herbicides. Residue Rev. 33:47-74.

23. Plimmer, J. R. 1974. The environmental dissipation of toxaphene. Abs. Am. Chem. Soc. 168th Natl. Mtg., Atlantic City, N.J.

24. Plimmer, J. R., P. C. Kearney, D. D. Kaufman, and F. S. Guardia. 1967. Amitrole decomposition by free-radical generating systems and by soils. J. Agric. Food Chem. 15:996-999.

25. Rosen, J. D., and M. A. Q. Khan. 1969. Properties of photoisomers of heptachlor and isodrin. J. Agric. Food Chem. 17:404-405.

26. Skipper, H. D., and D. T. Westermann. 1973. Comparative effects of propylene oxide, sodium azide, and autoclaving on selected soil properties. Soil Biol. Biochem. 5:409-414.

27. Southwick, L. M., G. H. Willis, P. K. Dasgupta, and C. P. Keszthelyi. 1976. The polarographic reduction of some dinitroaniline herbicides. Anal. Chim. Acta 82:29-35.

28. Udenfriend, S., C. T. Clark, J. Axelrod, and B. B. Brodie. 1964. Ascorbic acid in aromatic hydroxylation. I. A model system for aromatic hydroxylation. J. Biol. Chem. 208:731-739.

29. U.S. Environmental Protection Agency, National Bureau of Standards, National Science Foundation, and U.S. Energy Research and Development Administration. 1976. Symposium on Nonbiological Transport and Transformation of Pollutants on Land and Water: Processes and Critical Data Required for Predictive Description, May 11-13. National Bureau of Standards, Gaithersburg, Maryland. Program and Abstracts, unnumbered.

30. von Euler, H. 1916. Über die Lichtspaltung von Halogen-Essigsauren in Benzol und Äther. Chem. Ber. 49:1366-1371.

31. Zepp, R. G., N. L. Wolfe, J. A. Gordon, and G. L. Baughman. 1975. Dynamics of 2,4-D esters in surface waters. Hydrolysis, photolysis and vaporization. Environ. Sci. Technol. 9:1144-1150.

MICROBIAL DEGRADATION IN THE ENVIRONMENTAL HAZARD EVALUATION PROCESS

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ABSTRACT

An assessment of the environmental hazard of new chemical products must be made prior to their commercialization. Hazard evaluation should involve a systematic decision-making process to define and assess risk. Within the Detergents and Phosphate Division at Monsanto, this process has been evolving over the past few years and is based on the sequential accumulation of knowledge about new chemical products in the areas of environmental fate and aquatic and mammalian toxicology. Systematic and uniform procedures and test methods are used for this process. Four sequential phases of testing encompassing screening, predictive, confirmative, and monitoring studies are carried out parallel with commercial development of a new chemical so that appropriate business decisions based on environmental risk can be made periodically. Decisions to either continue testing, stop product development because of substantial risk, or stop environmental testing becuase of acceptable risk can be made at the various levels of testing. The mechanics of this process and the involvement of microbial degradation screening studies in the process are summarized.

INTRODUCTION

With the enactment of the Toxic Substances Control Act (TSCA) in 1976, industry will be required to conduct detailed environmental and mammalian safety studies prior to manufacture or new chemicals. A safety testing program should not be conducted in a random manner by running a battery of tests. Rather, some systematic procedure should be developed so that decisions as to the need for particular safety studies can be logically made. At Monsanto we have been developing a systematic environmental hazard evaluation process for new chemicals over the past few years (2, 3).

Environmental hazard evaluation is concerned with measurement of ecological toxicity and environmental fate. Standardized methodology for measurement of ecological toxicity, in particular aquatic toxicity, has progressed to a considerable extent primarily through interactions of various scientists and laboratories within ASTM. On the other hand, little if any uniform methodology exists in the area of environmental fate testing. Environmental fate studies are designed to establish mobility and persistence of chemicals in the environment. Among environmental fate studies, microbial degradation is often the major mode for disappearance of many chemicals in the environment and is perhaps the most important aspect of studies to measure persistence.

The present report gives an overview of the Monsanto Hazard Evaluation Process and describes how the microbial degradation screening studies employed at Monsanto fit into the overall process. Thoughts on the microbial degradation screening tests under TSCA are presented.

PRODUCT SAFETY EVALUATION

The overall safety evaluation program at Monsanto is depicted in Figure 1. During the initial research phases of a new chemical, the environmental scientists, consisting of specialists in the areas of mammalian and aquatic toxicology and environmental fate, review the product in terms of their prior knowledge of related materials, basic physical/chemical data, and anticipated volume/use/disposal patterns. From this evaluation, preliminary estimates of maximum environmental concentrations and potential toxicity are made. Screening studies for acute toxicity to mammalian and aquatic species and for environmental fate are then initiated. Based on these test results, combined with predicted environmental concentrations, the first major decision in the commercial development process can be made.

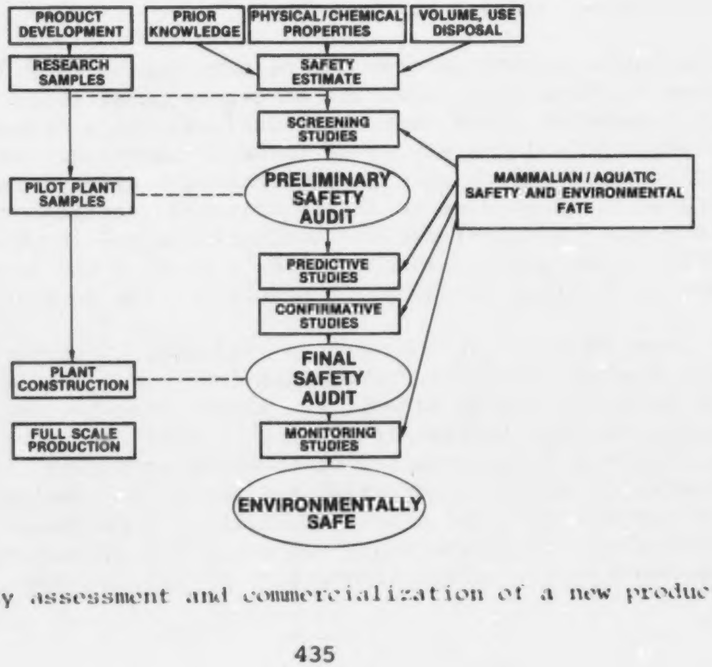

Figure 1. Safety assessment and commercialization of a new product.

If the decision is to proceed, a preliminary safety audit is held with our Department of Medicine and Environmental Health (DMEH), following which a comprehensive human and environmental safety assessment schedule is planned. This document defines the predictive and confirmative level studies which are needed to arrive at the proper decision on commercialization. Consideration is given not only to the intact parent material, but also to impurities, metabolites, and manufacturing effluents. At this time a commitment is usually made to scale-up the synthesis process to pilot plant quantities. Close interaction among the disciplines of the environmental safety group with commercial development activities is needed to assure that a project can be terminated as early as possible should adverse environmental properties inconsistent with intended use appear. Satisfactory completion of the confirmative tier triggers a final safety audit with DMEH in order to comprehensively review all environmental safety data and arrive at a final decision on commercialization and plant construction. Following commercialization, a monitoring program will assure that a product remains environmentally safe.

HAZARD EVALUATION PROCESS

Within the overall safety assessment program, the Detergents and Phosphate Division of Monsanto has been developing an environmental hazard evaluation process. This process, presented in Figure 2, is a systematic procedure for obtaining and interpreting environmental information on new products so that appropriate business decisions can be made at various points in the commercial development process. The basic premise under which this process has been developed is that aquatic toxicological data must be interpreted in relation to the expected or measured exposure concentration to aquatic organisms. Hazard evaluation, thus, involves a close interaction between the aquatic toxicology and environmental fate disciplines. The goal of this process is to specifically define the type of testing needed for a product and numerically indicate when sufficient testing has been done.

The environmental testing program can be arranged in four distinct and sequential phases or tiers (Fig. 2): the screening phase which hopefully under TSCA will consist of short-term, well defined, and standardized tests; the predictive phase in which a series of in-depth laboratory studies will increase our confidence in the chemical's environmental safety; the confirmative phase consisting of field studies to answer critical questions which have developed from laboratory studies; and the monitoring phase in which ultimate safety is verified under actual use conditions. Testing may stop at any tier or progress through all four depending on results of the studies.

As stated previously, aquatic hazard is evaluated by comparing aquatic toxicity to exposure concentration. From this integration of data, one of three decisions on hazard can be made, i.e., either stop testing because of unacceptable risk, continue testing in order to better define risk, or stop testing because the risk is acceptable. Laboratory screening and predictive studies are designed to yield a sequential refinement in knowledge of the expected exposure concentration and aquatic toxicity. Field-scale confirmative and monitoring studies will substantiate the environmental safety in nature under actual use conditions. The ultimate goal of this process is to organize the testing program so that time and dollars are not wasted in carrying out unnecessary tests. Our specific goal is to have numerical definitions of when to proceed with or stop testing.

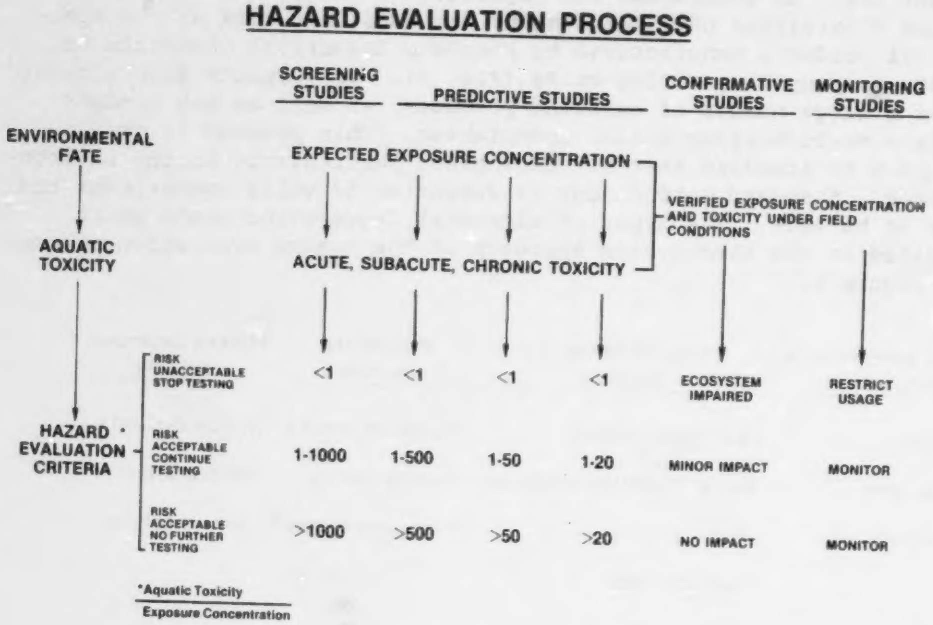
HAZARD EVALUATION PROCESS
SCREENING STUDIES
PREDICTIVE STUDIES
CONFIRMATIVE STUDIES
MONITORING STUDIES
ENVIRONMENTAL FATE
EXPECTED EXPOSURE CONCENTRATION
VERIFIED EXPOSURE CONCENTRATION AND TOXICITY UNDER FIELD CONDITIONS
AQUATIC TOXICITY
ACUTE, SUBACUTE, CHRONIC TOXICITY
HAZARD * EVALUATION CRITERIA
RISK UNACCEPTABLE STOP TESTING
<1
<1
<1
<1
ECOSYSTEM IMPAIRED
RESTRICT USAGE
RISK ACCEPTABLE CONTINUE TESTING
1-1000
1-500
1-50
1-20
MINOR IMPACT
MONITOR
RISK ACCEPTABLE NO FURTHER TESTING
>1000
>500
>50
>20
NO IMPACT
MONITOR
*Aquatic Toxicity / Exposure Concentration

Figure 2. Hazard evaluation criteria--whether more data are needed, enough data are available, or the project should be terminated.

For example, upon completion of screening level tests, expected exposure concentrations exceeding LC_{50} values (AT/EC<1) would usually indicate an unacceptable risk and testing along with commercial development of a new product would probably be terminated. Alternatively, if aquatic toxicity values are in 1000-fold excess of expected environmental concentrations, the risk from product use would be acceptable and no additional testing of the product would be indicated. However, AT/EC values of intermediate range would indicate more testing is required. These values should not be considered as fixed at this time nor to be applicable in all situations, for they are intended only as general guidelines and as a basis for discussion.

If continued testing is indicated we would progress into the predictive level of testing and at several points hazard could again be evaluated. It is important to note that as more data are accumulated on the product we are willing to accept a smaller safety margin between exposure concentration and effect concentration. Conversely, the safety margin between exposure concentration and effect level must be large before we are willing to invest in longer term, most costly studies. Business decisions are naturally easier when safety margins are either very high or low. Finally, if indicated, we would enter field scale testing to look for ecosystem effects and arrive at a decision on product use.

MICROBIAL DEGRADATION STUDIES

The process outlined represents an ideal toward which we are working. It is unfortunately much easier to define a hazard evaluation process than to fully implement one. To understand the magnitude of the evaluation process for a large and diversified chemical company one need only look at the spectrum of chemical products manufactured by Monsanto Industrial Chemicals Co., one of Monsanto Company's operating units (Fig. 3). To complete such a hazard evaluation for a large number of existing products, as well as new product candidates, is a multi-million dollar undertaking. This process is complicated by the lack of standard test methodologies, particularly in the environmental fate area. Standard methodology is essential if valid comparisons and judgments are to be made. The types of microbial degradation tests which might be required in the tier-system approach of the hazard evaluation process are shown in Figure 4.

Detergents & Phosphates Division	Specialty Chemicals Division	Plasticizer Division	Rubber Chemicals Division
BUILDERS	FUNCTIONAL FLUIDS	PHTHALATE ESTERS	ANTIDEGRADANTS
SURFACTANTS	WATER TREATMENT CHEMICALS	ADIPATE ESTERS	ACCELERATORS
ANTIMICROBIALS	PHARMACEUTICALS	PHOSPHATE ESTERS	CURING AGENTS
	FOOD ADDITIVES		
	FOOD INGREDIENTS		

Figure 3. A representative product spectrum of Monsanto Industrial Chemicals Company.

SCREENING	PREDICTIVE	CONFIRMATIVE
River Water	Natural Water Microcosms	Lake & River Ecosystem
Semi-Continuous Activated Sludge (SCAS)	Continuous-Flow Activated Sludge (CFAS)	Plant Uptake/Residue Studies
CO_2 Evolution	Contact-Stabilization Activated Sludge (CSAS)	
Aerobic Soils	Trickling Filter	
	Oxidation Pond	
	Anaerobic Soils	
	Anaerobic Digestor	
	Septic Tank/Soil	
	Plant Waste Treatability	
	Metabolism	

Figure 4. Typical studies to assess the microbial degradation characteristics of a new chemical.

This paper focuses only on the screening level of testing, an area where methodology is most widely published and the easiest to standardize. The type of screening studies used for a particular chemical should be dictated by its use/disposal patterns and its physical and chemical properties. If a chemical enters the environment primarily as a component of sewage, then screening tests involving sewage and natural waters should be used. Similarly, chemicals applied directly to the soil, such as pesticides, should be examined in soil systems and natural waters. Properties such as solubility, volatility, and adsorption may also affect the type of screening study. Microbial degradation screening studies in our laboratories (Fig. 5) have focused on aerobic environments and are designed to provide relative information on the primary and ultimate biodegradability of chemical products.

MICROBIAL DEGRADATION —
SCREENING TESTS

	DISCHARGE TO		
TEST	SOIL	SEWAGE	NATURAL WATER
CO_2 EVOLUTION	✓	✓	✓
RIVER WATER	✓	✓	✓
ACTIVATED SLUDGE		✓	
SOIL	✓		

Figure 5. Biodegradation screening studies which should be conducted as related to product discharge characteristics.

A. SEMI-CONTINUOUS ACTIVATED SLUDGE TEST

The 24-h SCAS test (5), as a simulation of secondary sewage treatment, has long been used in the soap and detergent industry. Studies of the activated sludge process suggest that the mean cell residence time is the most important control parameter. The typical 24-h SCAS test with its long mean cell residence time (≃30 d) provides maximum opportunity for biodegradation to occur. For SCAS studies at Monsanto we employ mixed liquor from a local domestic treatment plant and have applied two variations of this test depending on the solubility characteristics of the test material. For water-insoluble materials which adsorb on the sludge, 1500 ml, magnetically stirred bioreactors are used. Auxiliary stirring allows use of reduced aeration rates to minimize volatility of certain test chemicals and the large units provide enough activated sludge for daily extraction of adsorbed chemicals. For water-soluble materials, such as detergent surfactants and builders, we have found it advantageous to use smaller bioreactors with a 300-ml operating volume. Analytical methods for the latter materials involve primarily the aqueous phase so that depletion of the sludge does not become a problem.

One variable of the SCAS test, which should be further studied, is the nature of the supplemental waste which is fed along with the test chemical. Various types of synthetic waste have been employed, the most common being the nutrient broth/glucose/dibasic potassium phosphate waste recommended by the SDA (5). Other investigators have preferred natural sewage. Natural sewage provides a continuous input of an indigenous microbial population and organics more closely related to the real world. However, it is less convenient and the sewage may vary significantly in composition over an extended period of time. Natural sewage may also present an analytical background problem particularly when one is dealing with a multi-component chemical product. Synthetic sewage may provide greater convenience and reproducibility with minimal analytical background. It is, obviously, further removed from the real world than the natural sewage. We have some evidence that synthetic media give erratic results with some novel organic chemicals, possibly due to selection of a relatively narrow range of microbial species. We currently use both the SDA synthetic sewage and natural sewage, but some combination of natural and synthetic sewage might provide the basis for a single standardized test medium.

B. RIVER DIE-AWAY TEST

The river die-away (RDA) test by its simplicity and closeness to natural conditions has great appeal as a biodegradation screening test. The RDA test consists of exposing a low concentration of a test chemical to the natural microbial population in replicate river water samples. Changes in concentration of the chemical are monitored as a function of time by analyzing samples at selected intervals. While the variability in natural waters in both microbial count and composition would appear to be a serious problem in the development of standardized methodology, we feel that such a test has great usefulness as long as its limitations are recognized. We have carried out an extensive study of a number of the variables in a river die-away test (4). For a screening test water should be taken from a "typical" large river and used after allowing suspended solids to settle. Tests should be carried out at environmentally related concentrations consistent with the sensitivity of analytical methods. For most chemicals 10 ppb to 1 ppm would appear to be a reasonable concentration range. For water-insoluble materials the use of a spiking solvnet (acetone, ethanol, dimethyl sulfoxide, and methylene chloride) is permissible but its concentration should not exceed 20 µl/liter. As expected, temperature is extremely important; a logical choice for a screening test would be room temperature. Since agitation did not appear to have a significant effect, the use of quiescent conditions is a desirable simplication. Sterile controls are an obvious need in any river die-away test. We have utilized both heat and chemical sterilants, such as $HgCl_2$. A chemical sterilant should be used only if one is sure that there is no interaction with the test chemical. Perhaps the most important facet of a river die-away test should be the inclusion of several readily to moderately degradable reference chemicals to facilitate comparison of results. The development of a relative index might ultimately be possible.

C. ULTIMATE BIODEGRADATION--CO_2 EVOLUTION

Both the SCAS and RDA tests focus generally on primary biodegradation, the disappearance of the original chemical due to microbial action as evidenced by a specific analytical technique. Primary biodegradation measurements alone may not suffice because some chemicals which undergo rapid primary biodegradation may be converted to more persistent intermediates with different chemical and physical properties. The carbon dioxide evolution test has proved extremely useful in establishing the extent of ultimate biodegradation, the conversion to carbon dioxide, water, inorganic salts, and normal cellular products. We have utilized several test procedures to obtain this index of ultimate biodegradation, among these the Thompson-Duthie-Sturm (TDS) procedure (6) employing nine-liter bottles containing 6 liters of BOD water and acclimated bacterial seed, and the more convenient shake-flask system (6) developed in our laboratory. The shake flask (Fig. 6) basically consists of a 2-liter Erlenmeyer flask with a suspended reservoir for holding the carbon dioxide absorbent. The flasks are agitated on a rotary shaker. Periodic removal of the CO_2 absorbent for analysis and addition of fresh absorbent is carried out.

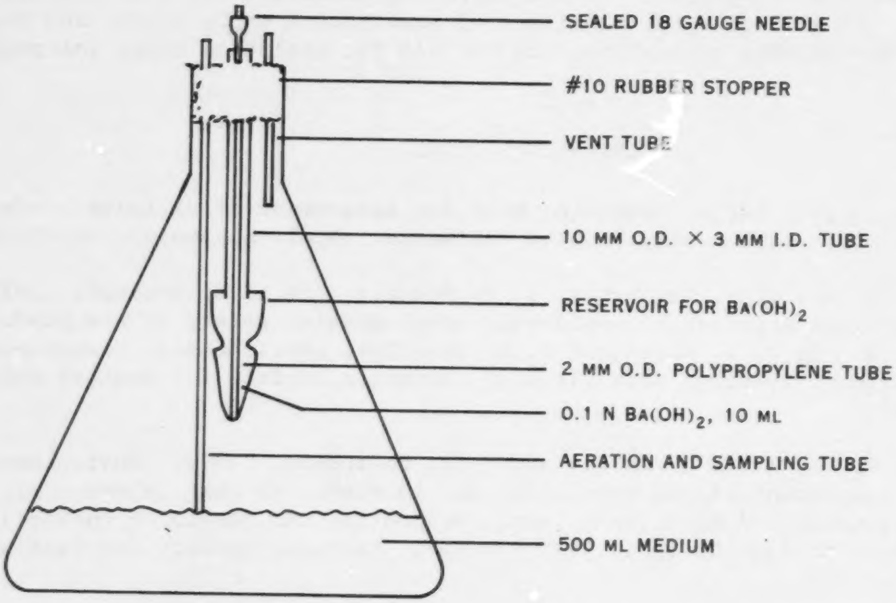

Figure 6. Shake flask for CO_2 evolution studies.

The rate and extent of carbon dioxide production are dependent on such factors as numbers and types of microorganisms, stability of the cells after growth, indigenous respiration, molecular structure, toxicity, and others. Determining the exact extent of mineralization necessitates a detailed material balance of initial and final concentrations of microbial cells, test material, metabolic intermediates, and CO_2. In large-scale screening studies these measurements are impracticable. Carbon dioxide evolution studies are useful in that values approaching the theoretical indicate essentially complete mineralization, whereas little or no CO_2 evolution may indicate resistance to biodegradation of the parent chemical or an intermediate. It is our opinion that carbon dioxide measurements present less ambiguity than oxygen uptake (BOD) measurements. Oxygen is utilized in many complex metabolic reactions and not simply in oxidization of the test chemical. Chemicals may also affect the endogenous respiration rate without undergoing degradation. Nitrification may also introduce uncertainty.

SCREENING TESTS UNDER TSCA

As many of you are aware the EPA is or will be recommending specific screening tests to assess microbial degradation under TSCA. It is our opinion that these tests should not be restrictive, but should be selected on the basis of the chemical's major route of entry into the environment and on its physical/chemical properties. In addition, the development of one good generalized biodegradation screening test which would be applicable to all organic chemicals should be developed. We are currently examining one such routine test system. It will give the simultaneous measurement of O_2 uptake and CO_2 evolution under aerobic conditions, and CO_2 and CH_4 evolution under anaerobic conditions.

LITERATURE CITED

1. Gledhill, W. E. 1975. Screening test for assessment of ultimate biodegradability: linear alkylbenzene sulfonate. Appl. Microbiol. 30:922-929.

2. Kimerle, R. A., G. J. Levinskas, J. S. Metcalf, and L. G. Scharpf. 1977. An industrial approach to evaluating environmental safety of new products, pp. 36-43. *In* F. L. Mayer and J. L. Hamelink, eds., Aquatic toxicology and hazard evaluation, ASTM STP 634. American Society for Testing and Materials.

3. Kimerle, R. A., W. E. Gledhill, and G. J. Levinskas. 1978. Environmental safety assessment of new materials, pp. 132-146. *In* John Cairns, Jr., K. L. Dickson, and A. W. Maki, eds., Estimating the hazard of chemical substances to aquatic life, ASTM STP 657. American Society for Testing and Materials.

4. Saeger, V. W., and E. S. Tucker. 1976. Biodegradation of phthalic acid esters in river water and activated sludge. Appl. Environ. Microbiol. 31:29-34.

5. Subcommittee on Biodegradation Test Methods of the Soap and Detergent Association. 1965, 1969. J. Am. Oil Chem. Soc. 42:986-993; 46:432-440.

6. Sturm, R. N. 1973. Biodegradability of nonionic surfactants: screening test for predicting rate and ultimate biodegradation. J. Am. Oil Chem. Soc. 50:159-167.

MICROBIAL DEGRADATION OF ORGANOCHLORINE COMPOUNDS IN ESTUARINE WATERS AND SEDIMENTS[1]

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ABSTRACT

Waters and sediments from an estuarine river near Savannah, Georgia, were incubated with various ^{14}C-labeled organochlorine compounds. The extent of microbial degradation of these compounds was determined by collecting the $^{14}CO_2$ produced after incubation periods of up to 4 d. A first-order rate expression fitted the degradation data for most compounds. Except for chlorophenol, with a half-life of 20 d in water, degradation was slow or absent for the chlorinated compounds in water. In sediments, high rates of degradation were observed for *p*-chlorophenol, trichlorophenol, trichlorophenoxyacetic acid and chlorobenzene with half-lives of 3, 23, 35 and 75 d, respectively. Degradation in sediments was very slow for hexachlorophene and DDE with half-lives of 290 and 1100 d, respectively. No degradation was observed for mirex, hexachlorobiphenyl and hexachlorobenzene in water or sediment. Large decreases in degradation rates were observed in the winter when water temperatures were 9-13°C.

INTRODUCTION

A wide variety of organochlorine compounds are introduced into estuaries from industrial effluents, sewage and the application of pesticides. The fate of these compounds in estuaries is affected by volatilization, photochemical degradation, sedimentation and biological degradation. This report deals with the rate of microbial degradation of selected organochlorine compounds in an estuarine area of the southeastern United States.

A number of studies have determined the metabolic pathways involved in degradation of organochlorine compounds using microbes isolated from fresh-

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water, marine and terrestrial sediments (3, 7, 9, 11, 13, and references cited therein). The microbial degradation of soluble organic compounds, such as glucose and amino acids, in estuarine water has been extensively investigated using ^{14}C-labeled compounds (5, 6, 15). This work has provided considerable insight into heterotrophic processes occurring in these waters. However, few studies have used ^{14}C-labeled organochlorine compounds to study the degradation of these pollutants in the estuarine environment, although there are reports on degradation of radiolabeled organochlorine compounds in sewage and terrestrial soil samples (2, 8).

The purpose of our study was to determine if ^{14}C-labeled organochlorines are degraded to $^{14}CO_2$ during incubation periods of up to 4 d. Degradation data were collected for a series of chlorinated benzenes and phenols in estuarine waters and sediments.

MATERIALS AND METHODS

Water and sediment were collected from the Skidaway River, an estuarine river located near Savannah, Georgia. Surface water samples were collected with a bucket and surface (upper 1 cm) sediment samples were taken at low tide with a spatula. Water temperatures averaged 13°C in the winter and 24°C in the summer, and salinity averaged 20 ppt (range 17-24 ppt) depending on rainfall and tidal influx.

Microbial degradation of the compounds in water was determined by adding ^{14}C-labeled compounds to 100 ml water samples in 250-ml flasks capped with silicon stoppers. At the end of the incubation period at the in-situ temperature, 0.2 ml of 1 N H_2SO_4 was added to the water and the respired $^{14}CO_2$ was collected in a center well containing a rectangle of Whatman No. 1 filter paper soaked with 0.4 ml of 2 N NaOH. After absorption of the $^{14}CO_2$ by the NaOH for 3 h, the filter papers were transferred to a second set of tubes equipped with $^{14}CO_2$ traps containing phenethylamine. One ml of 1 N H_2SO_4 was added to each tube and after 3 h the $^{14}CO_2$ traps were transferred to scintillation vials and counted with a liquid scintillation counter. The two steps for collection of $^{14}CO_2$ were necessary because of the small amount of the ^{14}C-labeled compounds volatilized and collected in the NaOH trap gave high blank values. Controls were water samples containing mercuric chloride at a concentration of 10 mg/liter. All samples were run in triplicate for each concentration and time interval.

Microbial degradation of organic compounds in sediment were determined by adding ^{14}C-labeled compounds to sediment-seawater slurries (1 g sediment and 50 ml of seawater in 125-ml incubator bottles capped with silicon stoppers). Controls were bottles containing mercuric chloride at a concentration of 10 mg/g sediment. At the end of the various incubation periods $^{14}CO_2$ was collected and counted as described above.

Radiolabeled compounds used included 1,4,5-trichlorophenoxy (1-^{14}C) acetic acid (54 mci/mM [Amersham]), Mirex-UL-^{14}C (5.8 mci/mM [CBN]), *p,p'*-DDE-UL-^{14}C (9.6 mci/mM [CBN]), phenol-UL-^{14}C (58 mci/mM [Amersham]), chlorobenzene-UL-^{14}C (4.2 mci/mM [CBN]), hexachlorobiphenyl, 2,4,5-2',4',5'-UL-^{14}C (9.06 mci/mM [NEN]), hexachlorobenzene-UL-^{14}C (23.7 mci/mM [NEN]), benzene-UL-^{14}C (107 mci/mM [Amersham]), 2,4,5-trichlorophenol-UL-^{14}C (9.8 mci/mM [CBN]), hexachlorophene-methylene-^{14}C (4.37 mci/mM [NEN]), *p*-chloro (U-^{14}C) phenol (3.0 mci/mM [Amersham]). The chemical name for mirex and DDE are dodecachlorooctahydro-1,3,4-metheno-2H-cyclobuta(c,d)pentalene and 2,2-*bis*(*p*-chlorophenyl)-1, 1-dichloroethylene, respectively.

RESULTS

MICROBIAL DEGRADATION IN WATER

Significant degradation of radiolabeled benzene, chlorobenzene, phenol, *p*-chlorophenol and *p*-chlorophenol to $^{14}CO_2$ took place in Skidaway River water samples (Fig. 1 and 2, Table 1). There was a lag before appreciable degradation occurred and the degradation for many compounds could be presented by the first-order equation

$$\frac{dc}{dt} = kC \text{ or } kt = \ln \frac{c_o}{c_o - c}$$

where k is the rate constant, c_o is the initial concentration expressed as micrograms per liter and c the concentration at time t. The rate of degradation of ^{14}C-benzene and ^{14}C-chlorobenzene has been plotted on a semi-log plot in Figure 2 to illustrate this relationship. Using the first-order rate expression, the rate constants were calculated using a least square analysis of the data. Phenol, chlorophenol and trichlorophenol had half-lives of 9, 20 and 690 d, respectively. Chlorobenzene had a half-life of 150 d while for benzene the half-life was 6 d. No degradation was observed for radiolabeled mirex, DDE, hexachlorobiphenyl, hexachlorobenzene and hexachlorophene. Thus, the number of chlorines on the molecule dramatically affected the rate of degradation.

TABLE 1. DEGRADATION OF RADIOLABELED COMPOUNDS IN SKIDAWAY RIVER WATER[a]

Compound	Water temperature (°C)	Rate constant, k (days^{-1})	Half-life $t_{\frac{1}{2}}$ (days)
Phenol	21	.079	9
p-Chlorophenol	9	.0014	490
p-Chlorophenol	21	.035	20
2,4,5-Trichlorophenol	21	.0010	690
Benzene	22	.11	6
Chlorobenzene	22	.0045	150
1,4,5-Trichlorophenoxy-acetic acid	22	.0005	1400

[a] Radiolabeled compounds were added to 100 ml water samples to give a concentration of 25 µg/liter. At the end of various incubation periods the $^{14}CO_2$ produced was collected and counted as a measure of the degradation. The rate constant for each compound was calculated from data fitted to a first-order rate expression.

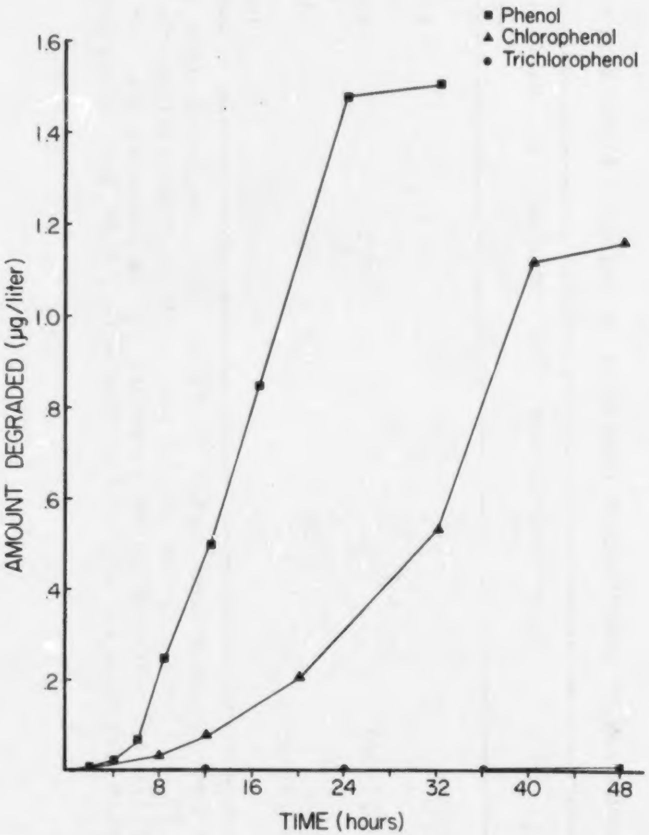


Figure 1. Degradation of phenol and chlorinated phenols in Skidaway River water. Concentration of the compounds was 25 μg/liter. Temperature was 21°C.

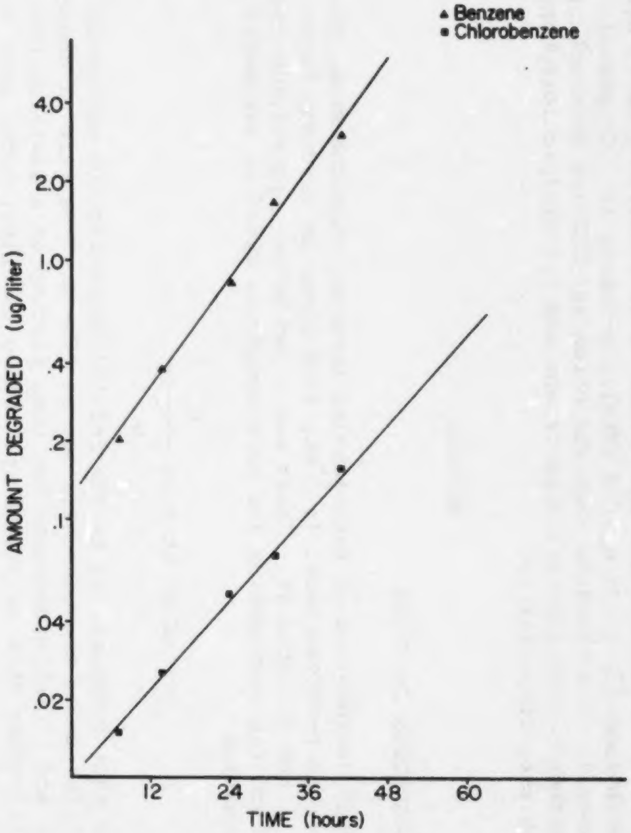


Figure 2. Log of the amount of ^{14}C-benzene and ^{14}C-chlorobenzene degraded with time in water from Skidaway River. The radiolabeled compounds were added to water samples to give a concentration of 25 μg/liter. Water temperature was 22°C.

The rates of degradation were low in the winter (water temperatures 9 to 13°C) so that the half-lives of *p*-chlorophenol in summer (21°C) and winter (9°C) was 20 and 490 d, respectively (Table 1). The observed decrease in degradation rate at longer incubation times (Fig. 1) presumably was due to a "bottle" effect which includes the effects of depletion of nutrients, pH changes, etc.

MICROBIAL DEGRADATION IN SEDIMENTS

The first-order rate expression appeared to also fit the degradation data for radiolabeled organochlorine compounds incubated with sediment samples. High rates were observed for *p*-chlorophenol, trichlorophenol, trichlorophenoxyacetic acid and chlorobenzene with half-lives of 3, 23, 35 and 75 d, respectively (Fig. 3 and 4, Table 2). Degradation was very slow for DDE and hexachlorophene with half-lives of 1100 and 290 d, respectively. No degradation was observed for mirex, hexachlorobiphenyl and hexachlorobenzene. Degradation was slow in the winter as illustrated in the degradation curves for trichlorophenol in winter and summer (Fig. 4). The half-life of this compound was 23 and 69 d during the summer (21°C) and winter (9°C), respectively (Table 2).

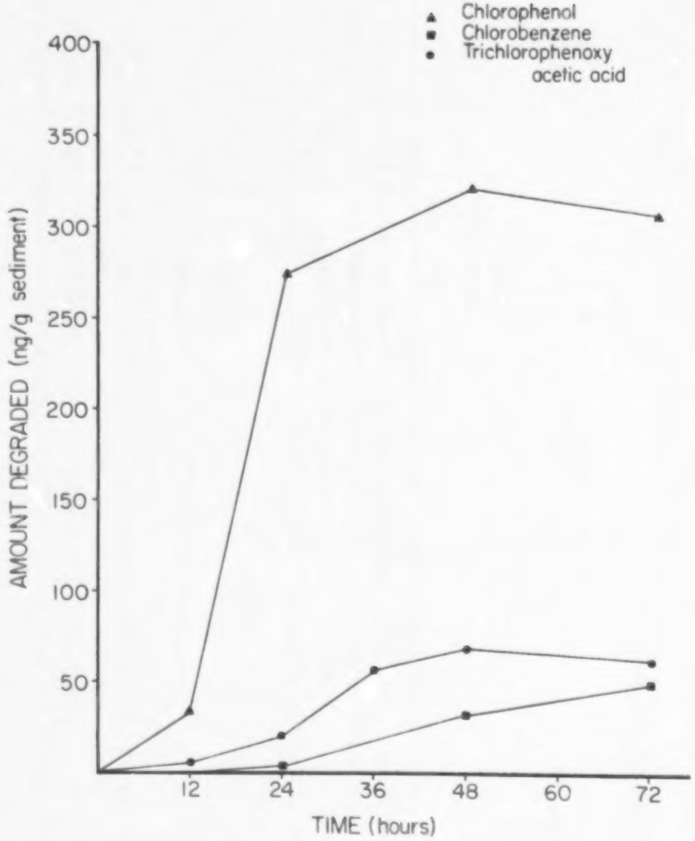
TABLE 2. DEGRADATION OF RADIOLABELED CHLORINATED COMPOUNDS IN SKIDAWAY RIVER SEDIMENTS[a]

Compound	Water temperature (°C)	Rate constant, k (days^{-1})	Half-life, $t_{\frac{1}{2}}$ (days)
1,4,5-Trichlorophenoxyacetic acid	9	.0012	580
1,4,5-Trichlorophenoxyacetic acid	21	.012	35
2,4,5-Trichlorophenol	9	.010	69
2,4,5-Trichlorophenol	21	.030	23
p-Chlorophenol	22	.23	3
Chlorobenzene	21	.0092	75
Hexachlorophene	22	.0024	290
p,p'-DDE	22	.0006	1100

[a] Radiolabeled compounds were added to sediment-water slurries to give a concentration of 2.5 μg/g sediment. At the end of various incubation periods the $^{14}CO_2$ produced was collected and counted as a measure of the degradation. The rate constant for each compound was calculated from data fitted to a first-order rate expression.

DISCUSSION

The methods described in this report, i.e., production of $^{14}CO_2$ from ^{14}C-labeled compounds, measured the potential of microorganisms in water and sediment to degrade various organochlorine compounds. Mineralization of 0.01% or less of a compound could be detected. After mineralization measurements of



'igure 3. Degradation of selected organochlorines in sediments from Skidaway River. Concentration of the compounds was 2.5 μg/g sediment. Temperature was 21°C.

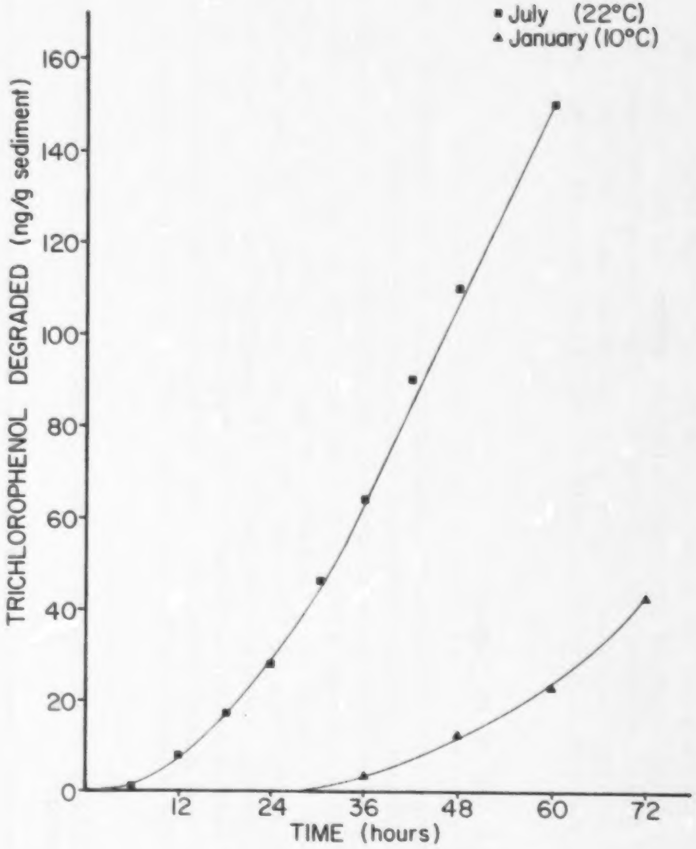


Figure 4. Amount of ^{14}C-trichlorophenol degraded with time at two different temperatures. The concentration at both temperatures was 2.5 μg/g sediment. Incubation temperatures were 10°C (January sediment sample) and 22°C (July sediment sample).

various model compounds, we can attempt to predict the persistence or nonpersistence of various classes of organochlorine compounds which enter the estuarine environment. Aromatic compounds with three chlorines or less showed some degradation in the water and rapid degradation in the sediment. More highly chlorinated compounds showed very slow or no degradation. The presence of a hydroxy group increased the degradation rate. The half-life in water of chlorophenol was 20 d while for chlorobenzene it was 150 d. Hexachlorophene with two hydroxy groups in addition to six chlorines had a half-life of 280 d whereas DDE had a half-life of 1100 d, and hexachlorobiphenyl showed no degradation.

No degradation of such highly chlorinated compounds as mirex, hexachlorobenzene and hexachlorobiphenyl could be measured in sediments as a long lag period is probably necessary before degradation occurs. Carson et al. (4) reported that after 12 years in the soil, mirex was partially degraded to kepone and related compounds. Hexachlorobenzene was not degraded after one year in soil containing 0.1 to 100 μg/g of the compounds (10). Since the methods used in our experiment measured only complete degradation to CO_2, any buildup of metabolites would not be detected.

The degradation curves resembled the typical log growth curve for bacteria (12). We speculate that initially there were low numbers of organochlorine-degrading bacteria in the water or sediment and growth of these microbes began after addition of the radiolabeled compound. The concentration of the pollutants under study in the water and sediment of Skidaway River were assumed to be very low since this river has no major pollutant input, except that resulting from boating traffic on this portion of the intracoastal waterway. Sediments from a similar estuary in South Carolina had concentrations of 1.8 and 3.0 ng/g sediment for DDE and polychlorinated biphenyls, respectively (14). The concentrations of these compounds added in our experiments were 25 μg/liter and 2.5 μg/g sediment for water and sediment, respectively.

Temperature and concentration of inorganic nutrients are important factors controlling biodegradation rates. Our studies indicated that degradation rates for all organochlorines studied were very low in both sediments and water during the colder periods of the year (December-March). Only the top layer of sediments were used to determine degradation rates. Deeper sediments in the study area are anoxic and no $^{14}CO_2$ was collected when subsurface sediments were incubated under nitrogen with the various organochlorine compounds. In anaerobic marine sediments, DDT can be dechlorinated to various products (1).

LITERATURE CITED

1. Albone, E. S., G. Eglinton, N. C. Evans, and J. M. May. 1971. Organic chemist's contribution to the Sabrina project. Mar. Pollut. Bull. 2:106-107.

2. Bartha, R., and D. Pramer. 1965. Features of a flask and methods for measuring the persistence and biological effects of pesticides in soil. Soil Sci. 100:68-70.

3. Bollag, J.-M. 1974. Microbial transformation of pesticides. Adv. Appl. Microbiol. 18:75-128.

4. Carlson, D. A., K. D. Konyha, W. B. Wheeler, G. P. Marshall, and R. G. Zaylskie. 1976. Mirex in the environment: its degradation to kepone and related compounds. Science 194:939-941.

5. Carney, J. F., and R. R. Colwell. 1976. Heterotrophic utilization of glucose and glutamate in an estuary: effect of season and nutrient load. Appl. Environ. Microbiol. 31:227-233.

6. Crawford, C. C., J. E. Hobbie, and K. L. Webb. 1974. The utilization of dissolved free amino acids by estuarine microorganisms. Ecology 55:551-563.

7. Focht, D. D., and M. Alexander. 1970. DDT metabolites and analogs: ring fission by *Hydrogenomonas*. Science 170:91-92.

8. Gledhill, W. E. 1975. Biodegradation of 3,4,4'-trichlorocarbanilide, TCC®, in sewage and activated sludge. Water Res. 9:649-654.

9. Heritage, A. D., and I. C. MacRae. 1977. Degradation of lindane by cell-free preparations of *Clostridium sphenoides*. Appl. Environ. Microbiol. 34:222-224.

10. Isensee, A. R., E. R. Holden, E. A. Woolson, and G. E. Jones. 1976. Soil persistence and aquatic bioaccumulation potential of hexachlorobenzene (HCB). J. Agric. Food Chem. 24:1210-1214.

11. Matsumura, F. 1974. Microbial degradation of pesticides, p. 129-152. *In* M. A. Q. Khan and J. P. Bederka (eds.), Survival in toxic environment. Academic Press, New York.

12. Pelczar, M. J., and R. D. Reid. 1965. Microbiology. McGraw-Hill, New York.

13. Rhead, M. M. 1975. The fate of DDT and PCB's in the marine environment, p. 137-159. *In* G. Eglinton (ed.), Environmental chemistry, Vol. 1. The Chemical Society, London.

14. Williams, R., and T. Bidleman. 1978. Toxaphene degradation in estuarine sediments. J. Agric. Food Chem. 26:280-283.

15. Wright, R. T., and N. M. Shah. 1975. The trophic role of glycolic acid in coastal seawater. I. Heterotrophic metabolism in seawater and bacterial cultures. Mar. Biol. 33:175-183.

APPLICATION OF A LABORATORY FRESHWATER LAKE MODEL IN THE STUDY OF LINEAR ALKYLBENZENE SULFONATE (LAS) BIODEGRADATION

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ABSTRACT

Laboratory lake models or microcosms were employed to determine the likelihood and pathways of LAS metabolism by microorganisms indigenous to a natural freshwater lake system. Microcosms were established by placing natural lake sediment and water in glass aquaria. In the unacclimated lake microcosms, treated initially to 5 ppm with C_{11}-LAS pure homologue, high pressure liquid chromatography (HPLC) analysis revealed primary LAS degradation to be complete within 18 d. The transient LAS biodegradation intermediates isolated by HPLC were identified by mass spectrometry as sulfophenyl carboxylic acids. These intermediates were themselves fully degraded within the four days following parent LAS disappearance. LAS biodegradation rates and pathways were similar for replicate microcosms. The lake microcosms described allow for the maintenance of essential interactions between aquatic and benthic microbial communities and, as such, are useful for assessing the biodegradation potential of the natural aquatic environment from which they were derived.

INTRODUCTION

The environmental acceptability of the detergent surfactant linear alkylbenzene sulfonate (LAS) is supported by numerous environmental fate and aquatic toxicology studies conducted with this material since its commercial introduction in the early 1960s. Primary and ultimate LAS biodegradability have been established in a variety of laboratory test systems employing municipal sewage, river water, or synthetic media (1, 8). In addition, field studies have confirmed LAS removal in sewage treatment plants (5, 6).

Biodegradation reduces the specific toxicity of commercial grade LAS to aquatic macroinvertebrates (*Daphnia magna*) and fathead minnows (*Pimephales promelas*) through preferential microbial metabolism of the more toxic homologues and isomers (3). Transient biodegradation intermediates are also less toxic to these species than the parent compound (3). Thus, LAS biodegradability in waste treatment facilities with concomitant toxicity reduction indicates that LAS does not pose a threat to aquatic life.

The research reported here focuses on the use of laboratory lake models or microcosms in the study of the LAS metabolizing capability of a freshwater lake microbial community. The lake microcosms, incorporating water, sediment, invertebrates, and microorganisms indigenous to the lake, are used to determine the biochemical pathways of LAS microbial degradation that are potentially operative in the natural lake ecosystem. Microcosms, as we have employed them, enable assessment of the biodegradability of synthetic organic compounds in a laboratory test constituted from representative environmental media and inocula under conditions that approximate the "real world," yet afford convenient operation and monitoring of the study. Our emphasis is on the use of lake microcosms as predictive tools for assessing LAS biodegradation rather than as simulated lake systems for detailed evaluation of the disposition of LAS among various model compartments.

LAS biodegradation has been shown to proceed through transient metabolic intermediates characterized as sulfophenyl carboxylic acids. Huddleston (2) showed the presence of sulfophenyl carboxylate metabolites in a shake flask culture treated with C_{12}-LAS. Swisher (8) successfully applied gas chromatography for detection of cyclized desulfonation products derived from short chain sulfophenyl carboxylic acids formed in river water die-away experiments. Leidner et al. (4) utilized mass spectrometry to confirm the identity of cyclized compounds obtained by desulfonation of sulfophenyl carboxylate metabolites. However, desulfonation/gas chromatography does not effectively detect carboxylic acid biodegradation intermediates of LAS unless the metabolite structures permit formation of 5 or 6 membered rings, and then only if the carboxylates are successfully dehydrocyclized. Consequently, Swisher et al. (9) developed and reported a high pressure liquid chromatography (HPLC) technique which enabled the direct detection of LAS metabolites from a river die-away study. The desulfonation step required for GC analysis was thereby eliminated, and the range of metabolite detectability was broadened.

In this work, we have refined the HPLC methodology of Swisher et al. (9) to monitor the formation and subsequent degradation of sulfophenyl carboxylic acid intermediates during LAS metabolism in lake microcosms. Gas chromatography/mass-spectrometry (GC/MS) has been utilized to confirm the identities of transient LAS biodegradation intermediates.

MATERIALS AND METHODS

MICROCOSM CONSTRUCTION

Lake microcosms were constructed using sediment and water from a spring-fed freshwater lake (Lake 34, Busch Memorial Wildlife Area, St. Charles County, Missouri). Three liters of lake sediment dredged from the littoral region of the lake, and 8 liters of lake water, were placed in 20-liter glass-covered aquaria. The microcosms were gently aerated and maintained under 300 foot-candle GE Daybrite fluorescent lighting on a 16/8 h light/dark cycle for 8 weeks prior to chemical treatment. This period allowed settling of particulate matter, stratification of the sediment into aerobic and anaerobic zones, and growth of aquatic macrophytes. Biological characterization of the microcosms, either before or after chemical treatment, was not attempted.

MICROCOSM TREATMENT AND SAMPLING

Replicate microcosms were treated with pure homologue C_{11}-LAS composed of 40% 5- and 6-phenyl, 20% 3-phenyl, and 19% 2-phenyl isomers. Commercial grade LAS consisted of a mixture of C_{10}-C_{14} LAS homologues with varying isomer distribution. However, only the more readily biodegradable C_{11} homologue was used in this study to facilitate confirmation of LAS biodegradation pathways. Twenty milligrams of LAS were added on each of two successive days, resulting in a total initial aquatic phase LAS concentration of 5 ppm.

One hundred milliliter composite water samples for methylene blue active substances (MBAS) and HPLC analyses were preserved with 5 mg mercuric chloride. Water temperatures and dissolved oxygen levels were determined with a dissolved oxygen meter (Model 54A, Yellow Springs Instrument Co.). Dissolved oxygen concentrations did not vary significantly from a concentration of 6.8-7.0 ppm.

SAMPLE ANALYSES

Fifty-milliliter water samples were concentrated for HPLC analysis by lyophilization and resolubilization in 1 ml of 10% methanol. After sonication and centrifugation, 50 µl of supernatant was injected into a Perkin-Elmer Series 3 HPLC equipped with a Waters Associates µ Bondapak C_{18} reverse phase ODS chromatography column. Detection of LAS and intermediates in the column effluent was accomplished by UV absorbance measurements at a wavelength of 223 nm using a Perkin-Elmer LC55 UV detector. A Hewlett-Packard 3385A computing integrator provided signal analysis capability.

The two solvents employed in the HPLC analysis were purified, filter sterilized water (Millipore Milli-Q-System) acidified to pH 3 with phosphoric acid, and UV grade methanol. Both solvents were degassed under vacuum prior to use. The HPLC was operated in linear gradient mode with methanol concentration in the eluent increasing from 10% to 100% at 5%/min. Total solvent flow rate was 1.5 ml/min.

Major, recurring HPLC peaks were collected for GC/MS determination of molecular weight and structure. Sulfophenyl carboxylic acid biodegradation intermediates were converted to free acids by passing the LC cuts through a cation exchange column (Dowex 50W-X8). Methyl derivatives, formed by reaction of concentrated samples with diazomethane, according to the procedure of Schlenk and Gellerman (7), were dissolved in 100 µl hexane for GC/MS analyses. The GC column was packed with 3% OV 101 on 100-120 mesh Gas Chrom Q and operated following a temperature program of 150-300°C at 16°C/min. Helium flowing at 30 ml/min was the carrier gas for electron impact (EI) GC/MS; chemical ionization (CI) GC/MS utilized methane carrier at a 10 ml/min flow rate. A Hewlett-Packard 5928A mass spectrometer coupled with a Hewlett-Packard 5934A data system generated mass information.

STANDARD MATERIALS

HPLC and GC/MS techniques for LAS biodegradation intermediate separation and identification were developed using model LAS metabolites. Disodium salts of the sulfophenyl carboxylic acids 3-sulfophenylbutyric acid, 4-sulfophenylvaleric acid, 3-sulfonphenylheptanoic acid and sulfophenylundecanoic acid were prepared and purified prior to this study as described by Swisher et al. (9) and Kimerle and Swisher (3). Reference solutions containing known amounts of the individual sulfophenyl carboxylates were employed to assess the capability of the sample preparation and HPLC methods for recovery of the intermediates from the microcosm water matrix.

RESULTS AND DISCUSSION

The general progress of LAS biodegradation over the test period is indicated by the results of MBAS analysis of water samples as shown in Figure 1. MBAS disappearance rates were similar for replicate microcosms.

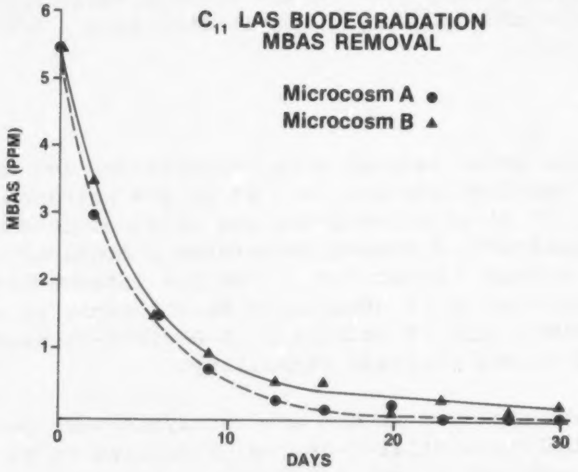

Figure 1. MBAS removal from the microcosm aquatic phase during biodegradation of C_{11} LAS: ● = Microcosm A, ▲ = Microcosm B.

Our HPLC technique effectively separated and detected sulfophenyl carboxylic acids as well as parent LAS. Figure 2A shows the chromatogram of 50 µl of a standard solution containing the 4 model intermediates as well as pure homologue LAS of alkyl chain lengths 10, 12, and 14. Each peak represents 1 µg of material. The model intermediates emerge in order of increasing chain length, 4, 5, 7 and 11. The last 3 peaks of the chromatogram correspond to LAS also eluting in order of increasing chain length. Methanol absorbs UV light at the 223 nm wavelength used for detecting the benzene ring of LAS and its metabolites. Consequently, the chromatogram baseline rises as the solvent becomes more concentrated in methanol.

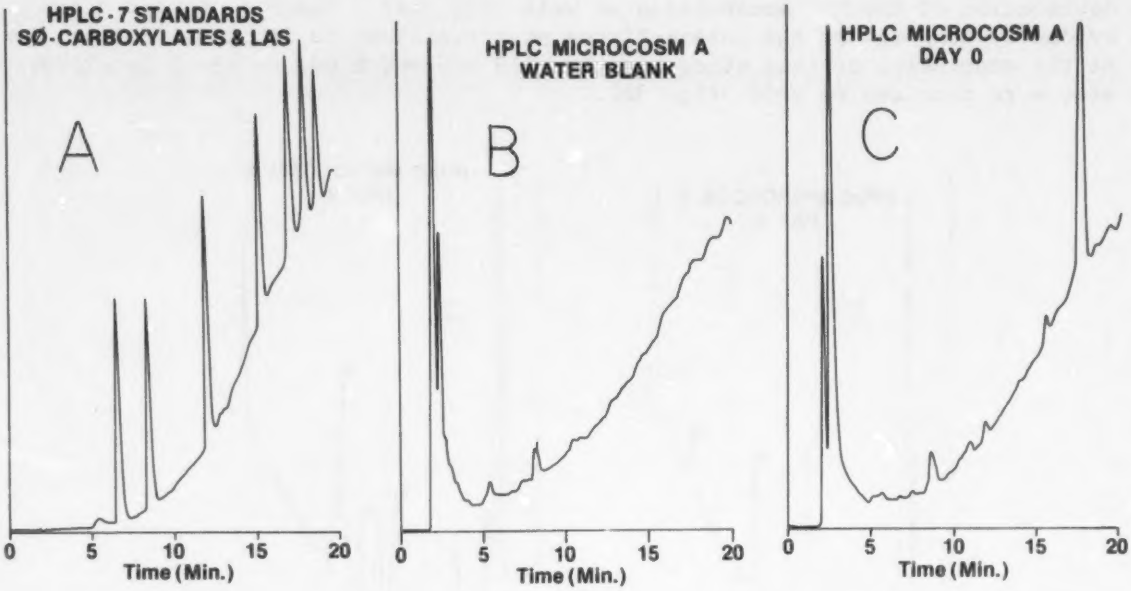

Figure 2. HPLC chromatograms of LAS pure homologues and sulfophenyl carboxylate metabolites: A--standard solution, 1 μg each C_4, C_5, C_7, C_{11} sulfophenyl carboxylic acid and C_{10}, C_{12}, C_{14} LAS; B--concentrated microcosm water blank; C--concentrated microcosm water sample day 0.

Model intermediate recovery studies indicated that 70% of the sulfophenyl carboxylates present in a microcosm water sample were actually measured by HPLC when our sample concentration scheme was used. Only 45% of LAS present in a microcosm sample could be recovered by this same technique. Our inability to completely recover LAS and sulfophenyl carboxylate intermediates probably resulted from difficulties encountered in fully resolubilizing lyophilized water samples in methanol. The lower detection limit for metabolites in water by this method was 50 ppb.

The chromatogram of a concentrated microcosm A water sample taken before treatment with LAS is shown in Figure 2B. The large peaks eluting between 2 and 3 min are thought to be nitrates which absorb light at this UV wavelength.

Figures 2C and 3 reveal the course of LAS biodegradation, and of LAS metabolite formation and disappearance, during a 30-d test in microcosm A. The parent C_{11}-LAS elutes at 17.5 min and is the only material detected by HPLC on the day of treatment (Fig. 2C). After 2 d, LAS has begun to degrade and 3 major metabolite peaks appear in the chromatogram. (A, B and C with retention times t_r = 8.5, 11.7 and 15.3 min, Fig. 3A). By day 9 (Fig. 3B), peak C is no longer detected. Since LAS is still present in the system, peak C is probably a short-lived, longer chain sulfophenyl carboxylate that is rapidly degraded by acclimated microorganisms present in the system after the first week of operation. Peaks A and B of day 2 are joined by significant amounts of material giving rise to the peaks labeled D, E, F, and G. At day 13, LAS primary biodegradation is nearly complete and HPLC analysis clearly shows substantial degradation of the LAS metabolites as well (Fig. 3C). Peaks F, B, and G are evidently composed of the intermediates most resistant to microbial metabolism. At the conclusion of this study, neither LAS nor any biodegradation intermediates were detected by HPLC (Fig. 3D).

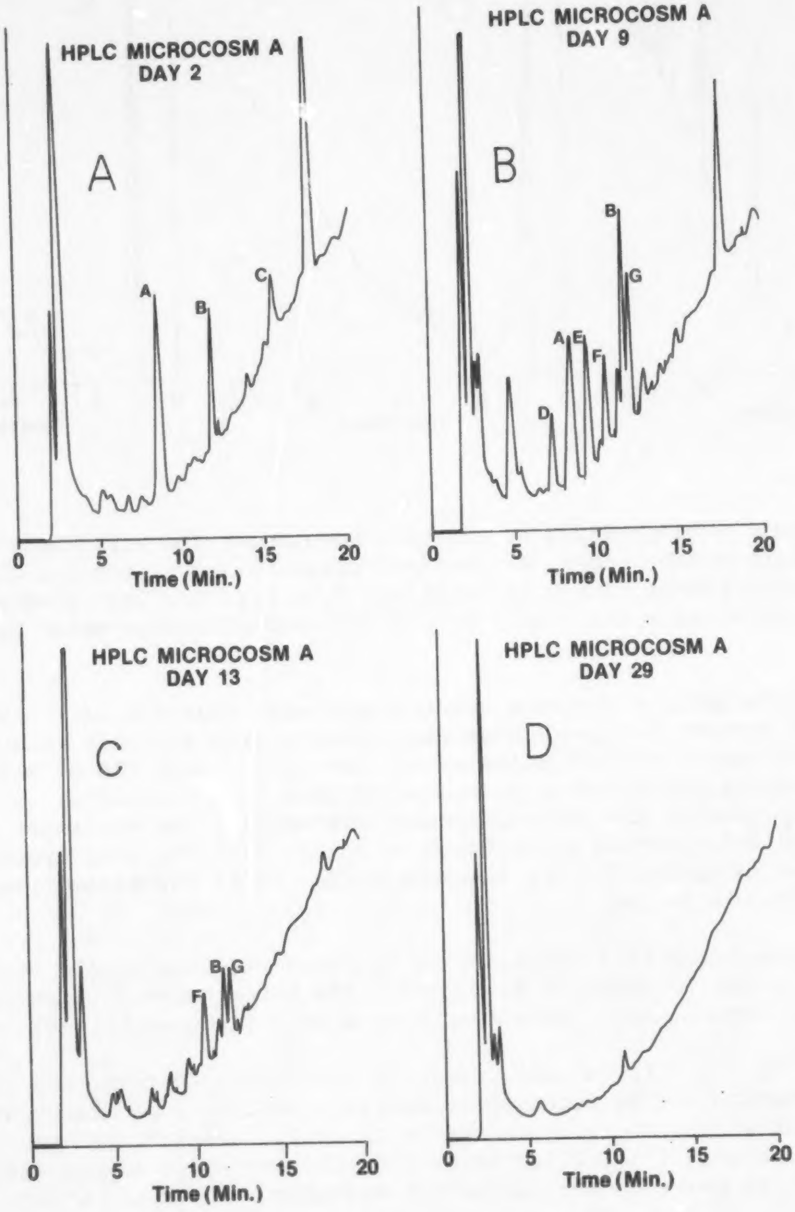

Figure 3. HPLC chromatograms of C_{11} LAS pure homologue and metabolites in concentrated microcosm water samples. A--Day 2; B--Day 9; C--Day 13; D--Day 29.

Throughout this investigation, HPLC analyses indicated that both the pathways and rates of LAS and intermediate biodegradation were similar for replicate microcosms A and B. Major metabolite concentrations and HPLC peak retention times for the two systems were nearly identical on any given sample date. Chromatograms of samples from microcosms A and B taken on day 6 are compared in Figure 4.

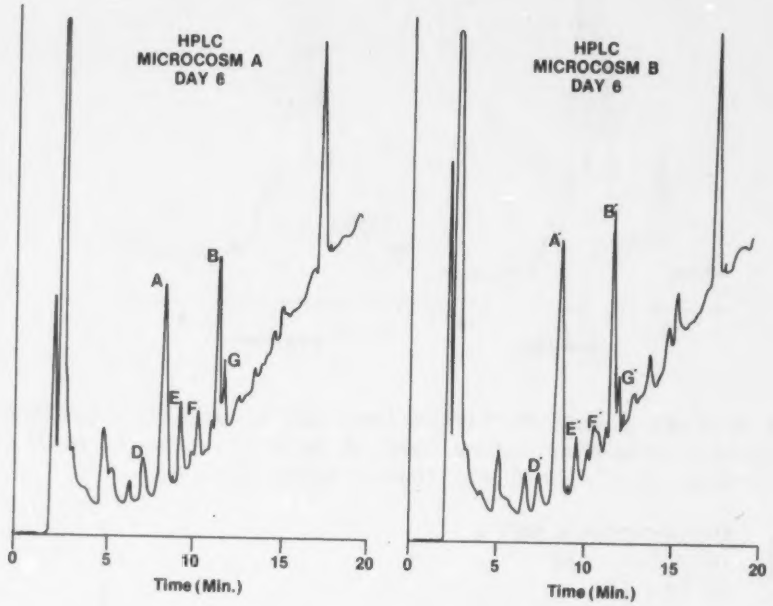

Figure 4. HPLC chromatograms of concentrated water samples from replicate microcosms, Day 6.

Major and recurring metabolite peaks were collected and analyzed by GC/MS to establish biodegradation intermediate identities. In general, gas chromatography of HPLC peaks showed each peak consisted of several components. CIMS confirmed the identity of these components as sulfophenyl monocarboxylic acids with carbon chain lengths ranging between 4 and 8 carbon atoms. No dicarboxylic acid intermediates were found. EIMS was employed to distinguish between the various isomers that must exist among the intermediates. Interpretation of results from these fragmentation studies is not complete and is not presented here.

Gas chromatographs of methylated HPLC peaks B/B' (t_r = 11.70 min) from day 6 samples of microcosms A and B are shown in Figure 5. Both HPLC peaks consist of at least 4 major components separated by GC. GC retention times are the same for samples from replicate microcosms.

Figures 6 and 7 show the CI mass spectra for two of the four GC peaks in Figure 5. The mass spectrum for the 4.0 min peaks reveals a parent species with molecular weight 301. This corresponds to the protonated dimethyl derivative of a C_6 sulfophenyl monocarboxylic acid. The secondary peak at molecular weight 269 results from the loss of methanol from the protonated parent molecule, a characteristic process in CIMS analysis.

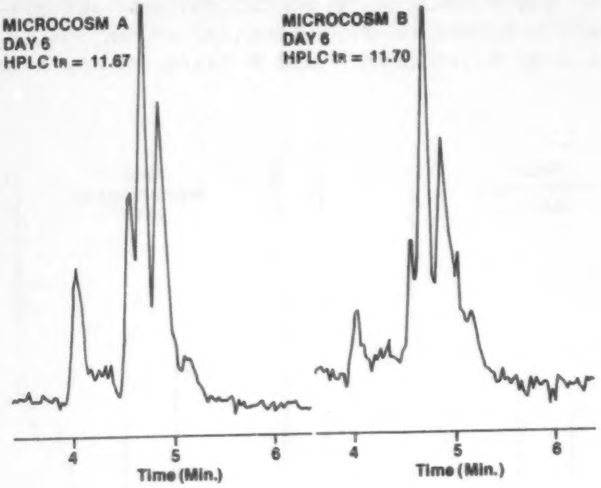

Figure 5. Gas chromatographs of derivatized LAS metabolites isolated from replicate microcosm water samples by HPLC. Day 6, HPLC peak retention time 11.67/11.70 min (Peaks B/B', Fig. 4).

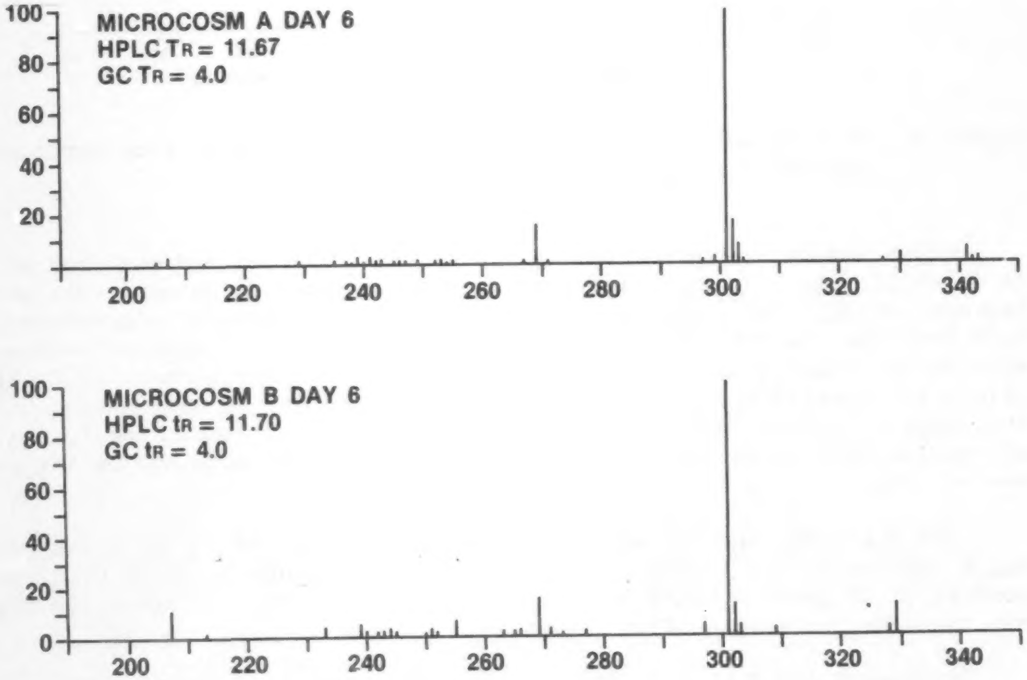

Figure 6. CI mass spectra of derivatized LAS metabolites from replicate microcosms A and B. (HPLC peaks B/B', Fig. 4; GC peak retention time 4.0 min, Fig. 5).

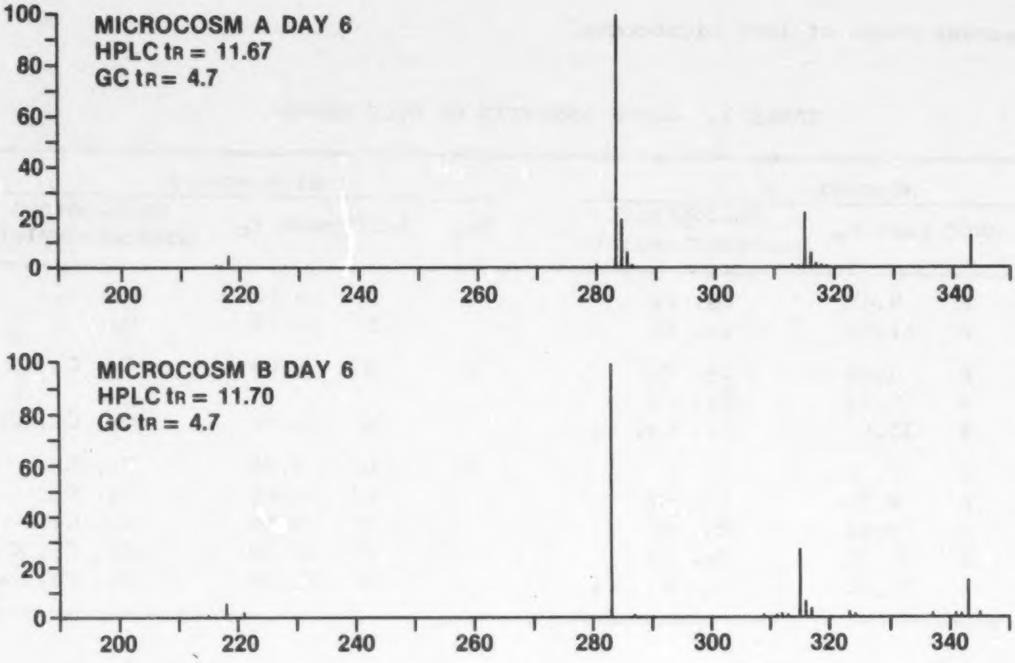

Figure 7. CI mass spectra of derivatized LAS metabolites from replicate microcosms A and B (HPLC peaks B/B', Fig. 4, GC peak retention time 4.7 min, Fig. 5).

In Figure 7, the parent molecule has a molecular weight of 315 indicating the protonated dimethyl derivative of the C_7 monocarboxylate. Loss of methanol from this species is again revealed by the strong peak at 283, while the signal at molecular weight 343 is generated by the product of ethane radical $(C_2H_5^+)$ addition to the parent molecule. Table 1 summarizes the results of GC/MS analysis of HPLC peaks.

The environmental transport and transformation of synthetic organic compounds such as LAS are effected by numerous physical, chemical and biological processes. These processes are frequently isolated in laboratory experiments to establish the relative importance of a particular pathway in determining the fate of the compound in the environment. In natural ecosystems, however, these pathways operate simultaneously. Thus, model ecosystem or microcosm studies are valuable as integrative laboratory tests that allow for the maintenance of process interactions characteristic of the natural environment.

Previous work concerning the environmental fate of LAS has shown microbial metabolism to be the most important route for the degradation of LAS and its metabolites in aquatic systems. Indeed, the LAS molecule was originally designed to function as a readily degradable alternative to its predecessor, the branched chain alkylbenzene surfactants. Consequently, while we employed a test system in which a variety of transformation processes were potentially operative, our analysis focused exclusively on the biodegradation of LAS in the aqueous phase of lake microcosms.

TABLE 1. GC/MS ANALYSIS OF HPLC PEAKS

Microcosm A				Microcosm B			
Day	HPLC peak	t_r	Sulfophenyl monocarboxylate	Day	HPLC peak	t_r	Sulfophenyl monocarboxylate
2	A	8.45	C_5, C_6	2	A'	8.52	C_5, C_6
	B	11.69	C_7, C_8		B'	11.74	C_7
6	A	8.61	C_5, C_6	6	A'	8.63	C_5, C_6
	E	9.56	C_5, C_6				
	B	11.67	C_6, C_7, C_8		B'	11.70	C_6, C_7, C_8
9	D	7.35	C_4	9	D'	7.36	C_4, C_5
	A	8.50	C_5, C_6		A'	8.62	C_5, C_6
	E	9.52	C_5, C_6		E'	9.56	C_5, C_6
	F	10.58	C_5, C_6		F'	10.58	C_5, C_6, C_7
	B	11.70	C_6, C_7, C_8		B'	11.70	C_6, C_7, C_8

Direct HPLC analysis of concentrated microcosm water samples clearly revealed the biodegradation of the C_{11} LAS molecule and the formation of transient sulfophenyl carboxylate metabolites. LAS and its biodegradation products in the aqueous phase were metabolized to levels undetectable by our analytical procedures within three weeks following initial chemical treatment of the unacclimated microcosms.

Sulfophenyl carboxylate biodegradation intermediates had both even and odd numbers of carbon atoms in the carboxylate side chain as shown by GC/MS determinations of metabolite molecular weights. While the latter is consistent with the usually proposed pathway for metabolism of LAS (ω,β oxidation) (8) the presence of intermediates with even side chains derived from C_{11}-LAS indicates that an alternative oxidative route, perhaps involving α-oxidation and cleavage, may be utilized by freshwater microbes in degradation of LAS.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Gledhill, W. E. 1975. Screening test for assessment of ultimate biodegradability: linear alkylbenzene sulfonates. Appl. Microbiol. 30: 922-929.

2. Huddleston, R. L., and R. C. Allred. 1963. Microbial oxidation of sulfonated alkylbenzenes. Dev. Ind. Microbiol. 4:24-38.

3. Kimerle, R. A., and R. D. Swisher. 1977. Reduction of aquatic toxicity of linear alkylbenzene sulfonate (LAS) by biodegradation. Water Res. 11:31-37.

4. Leidner, V. H., R. Gloor, and K. Wuhrmann. 1976. Degradation kinetics of linear alkylbenzene sulfonates. Tenside Detergents 13:122-130.

5. Mann, A. H., and V. W. Reed. 1971. Biodegradation of synthetic detergents evaluation by community trials: I. Linear alkylbenzene sulfonates. J. Am. Oil Chem. Soc. 48:588-594.

6. Renn, C. E., W. A. Kline, and G. Orgel. 1964. Destruction of linear alkylate sulfonates in biological waste treatment by field study. J. Water Pollut. Control Fed. 36:864-879.

7. Schlenk, H., and J. L. Gelerman. 1960. Esterification of fatty acids with diazomethane on a small scale. Anal. Chem. 32:1412-

8. Swisher, R. D. 1970. Surfactant biodegradation. Marcel Dekker, Inc., New York.

9. Swisher, R. D., W. E. Gledhill, R. A. Kimerle, and T. A. Taulli. 1976. Carboxylated intermediates in the biodegradation of linear alkylbenzene sulfonates (LAS). 7th Int. Cong. Surf. Act. Subst. Paper No. 117,D2, Moscow.

DISCUSSION

METHODOLOGY

RAYMOND: We have touched on many of the aspects of what you presented to us today, Dr. Plimmer, in the first three days of the Workshop. Do you have any specific recommendations for us as far as what we can do in practical terms to set up our systems for studying biodegradation, taking into account all of these very important physical and chemical factors you have been discussing?

PLIMMER: I think the first thing is to be aware of them. Obviously when we start the study of a pollutant we model our study on previous work from the biological point of view, but it is necessary to know the physical and chemical properties of the molecule under study. You can make basically a set of predictions on those grounds. Before you start you need to know the solubility, and vapor pressure of the compound, and if they are not known, it is just as well to try to obtain these data. A critical examination of the behavior of structural analogies recorded in the literature will help you to determine whether the compound will be very susceptible to degradation under any of the chemical conditions that you set up in your particular biodegradation study.

RAYMOND: In our Task Group, we have been discussing these factors. We probably have decided that one of the initial steps in any protocol or testing for biodegradation should include very thorough examination of the chemical and physical properties of the compound.

PLIMMER: The other thing to remember is that a full study of a chemical reaction, such as hydrolysis, may be as costly as a biodegradation study. If you want to satisfy the criteria of the physical chemist, you can spent a lot of time and effort on studies of rates of hydrolysis. In this last situation, I have discussed that in a homogeneous medium. I still ask the question: What are the implications of our data for reactions at interfaces? I think this is where a lot of the action is.

RAYMOND: Of course, all of us are faced with the practicality of setting up a system, and we don't have time to go through all this very elegant detailed examination. I am just wondering, is there a test? Is there a method that we can apply to a particular chemical to give us some insight fairly rapidly into physical/chemical factors like we have in biodegradation studies?

PLIMMER: I think one of the most useful factors is the partition coefficient, between octanol and water. That is a very useful piece of data for many compounds. In terms of adsorption, chromatographic techniques will tell you a great deal about adsorptive properties. Those are the studies which you will have to conduct to identify products. Those are two areas which will give you a rapid insight into those physical properties which may be important.

MATSUMURA: You mentioned the octanol partition coefficient but there are so many variations among the research groups in the values. For instance, when you compare the Mirex partition coefficient between Metcalf's group and Freeze's group, there is a 1000-fold difference in the value. I wonder if you have any suggestions to standardize the technique?

PLIMMER: I have no suggestions for standardizing the technique, but I suggest that it should be standardized. That is one of the things which you ought to be able to measure with a reasonable degree of reproducibility from lab to lab. It may mean, in fact, doing one of those physico-chemical studies that I suggested earlier that you don't do, controlling all factors, trying to achieve equilibrium and so on. It's very difficult with compounds that are not very soluble to measure or to discuss their solubility. What is meant by solubility? This is a major hurdle in this area.

GARNAS: I like the discussion you got into on the use of various sterile controls in running experiments. I have often found in my involvement with microbiologists (Hap or Al) that the sterile control is the answer to nonbiological degradation--it's the physical/chemical control. I was wondering if you wanted to expand a bit on the use of various sterilizing methods that might be more acceptable or a range of methods. For example, autoclaving is not residual; you can get contamination again. The use of formalin or various other types of chemosterilants can alter the sediment. Would you like to expand on this a bit, please?

PLIMMER: Well, the methods I discussed here, ethylene oxide and propylene oxide sterilization obviously have least effect on some of the factors that we were discussing, such as the chemical residues peroxidase, activity and so on. There are suggestions that irradiation sterilization might be useful but I criticize this technique because many of the reactions in the soil may be due to unpaired electrons. If you started irradiating your soil you may generate many more free-radicals. The radical content of the soil may be increased by the irradiation. Obviously, heat sterilization is going to have the other effect. I don't know whether Don Kaufman has any comments; he has been more recently involved in this work than I have. I think it remains a problem and that is about where we stand at present.

KAUFMAN: There are a number of methods for sterilizing soil. I think the point is that we cannot rely on any one method as a sole criterion to determine whether biodegradation is the process or something else is the process. This is really our interest. We were looking at a number of chemicals which were claimed in the literature to be biodegradable solely on the basis that no degradation occurred in autoclaved soil. That is not an adequate criterion for determining whether there is biodegradation. By destroying the soil with autoclaving, you destroy the chemical reactions which do have an appearance of being a microbiological reaction. This is one of the problems that I think we faced, and one place where microbiologists can contribute to more accurate information on chemicals in the environment. The danger that I see, you can go the other way and suddenly end up with an experiment so large with various sterilization techniques that your non-sterile or real-world system is off in one corner of the experiment.

PLIMMER: As a chemist, I ask the same question about aquatic environments. If you have a strongly anaerobic system and a redox potential which is extremely low, you add a reactive chemical and that chemical is reduced. Is that, in fact, metabolism? Insofar as the chemist is concerned, it is just another transformation. But it is not strictly necessary for the biological system; the microbes are not deriving any benefit from it.

STERN: During the past few days and evenings I have sat in with two or three task groups. We have hit on a problem and bounced away from it because it was a complex one. From a regulatory standpoint we seem to have a "Catch 22" situation, particularly in view of what you have told us this morning. It is simply this: we are concerned about the pollutants that actually get into the environment and form part of the exposure picture that we have to deal with. Some of the physical/chemical parameters that you suggest measuring can only be measured on pure compounds. We have to deal with formulated materials with associated impurities, and I am quite sure these impurities are going to affect profoundly the results you get in physical/chemical testing. I wonder if you would have any "pearls of wisdom" for us that would help resolve this problem?

PLIMMER: I do not advocate going through all these procedures as part of a protocol. All I am doing is pointing out various considerations and playing the part of the chemist as devil's advocate. What you have raised is another point that you can't ignore. In the complexity of the real-world situation, you may have a formulated material present as an emulsion, the surfactant may or may not change the bioavailability of the active ingredient. These considerations force us to examine interactions between two materials and the problem of interfaces. I really don't know what the answer is, whether or not there can be simple protocols for measuring factor X to give all the answers. That would be rather nice, but at present there is no simple answer. It is a matter of organizing meetings of this sort to bring chemists and microbiologists together and hammering out the issues once more.

CLESCERI: Dr. Gledhill, do I understand that in your semi-continuous activated sludge process, when you are looking for the disappearance of your test chemical, that you look only in the liquid phase? Or do you also look to see what is associated with the sludge?

GLEDHILL: In the screening studies of the products which are very water soluble in our laboratory we look primarily at the liquid phase or dissolved organic carbon in the effluent. For products that are absorbed to the sludge we do look at both phases. Again, this is just at the screening level.

CLESCERI: Certainly it is just as important really to know what stage is associated with the sludge.

GLEDHILL: It is, but when you consider in our lab we are looking at 50 or 60 different compounds at one time, it is difficult to look at the sludge for the compounds. Usually with new compounds, we don't have analytical methods.

CLESCERI: Yes, that is a problem. Is the sludge at all pre-conditioned as far as your test compounds are concerned?

GLEDHILL: Yes, in the activated sludge test, it is a 24-hour cycle. However, this is continuous for a period of up to 15 weeks with the test compound being added every day, so there is time for acclimation.

DAUGHTON: In your toxicity testing at the screening level do you take into consideration synergistic effects at all in any environment?

GLEDHILL: For toxicity studies at this level, we look at pure cultures. If it's microbial, for instance algae, we look at several pure cultures to find out what level we get an EC_{50} effect.

DAUGHTON: In particular, what I was concerned with was synergism of breakdown products with the parent compound. I was wondering if perhaps it would be possible to directly couple a toxicity study with a degradation study.

GLEDHILL: Yes, we do this. Again, this is screening, not a test. This is looked at in the predictive level. For more detailed tests, we do run biodegradation studies looking at effluent, and the toxicity of the degradation products.

DAUGHTON: In your shake flask with CO_2 traps, do you really call that CO_2 evolution, or evolution of labelled volatiles?

GLEDHILL: We use that mainly for new chemicals for which we don't have analytical techniques. We call it carbon dioxide evolution because we don't use labelled compounds.

FLOODGATE: I was a little surprised when you said that you preferred CO_2 evolution to O_2 uptake as a means of measuring biodegradation. Would you say that was true in the sea as well when you have a strong buffer system or carbonate/bicarbonate buffering system?

GLEDHILL: In marine systems, I don't know. I would expect you would have problems there in determining CO_2 evolution.

FLOODGATE: We tend to reject the idea of using CO_2 evolution, because the whole system is buffered by bicarbonate/carbonate system. When you are starting to deal with a buffering system, you're essentially altering the whole system.

GLEDHILL: No, again, we don't even consider these systems when you get a new chemical for the first time in the lab. We are doing screening studies. As we get into the environment, into real systems, then we need specific methodology and we use labelled compounds.

GLOODGATE: Fine, that suits me now, thank you.

ROGERS: You talked about the stage testing of your chemicals. In your first screening tests, you mentioned three levels. One of the numbers you used in your first level was a thousand. What I was wondering, if a chemical falls in this range of being over a thousand, and you decide that it is worth producing on other decision levels, do you go back and put it through a complete testing range again?

GLEDHILL: Well, I think that there would be situations where you would want to do this, especially if there was a potential for a large volume product.

ROGERS: For instance, in the synergistic effect he was talking about, you may put it through the whole testing scheme, yet you may never detect something even though the screening stage number could be around ten thousand.

GLEDHILL: One of those things that we need to have some logical decision on is when do we stop testing and when do we go on? As I said, this number of a thousand really doesn't mean anything now; this was one number that has been considered.

BETTINGER: One of the interests that many environmental departments and companies have many times when you go to scale-up is how will these compounds affect your treatment system? Therefore, you usually examine the effluent waste from that kind of plant in an aquatic toxicological test. You might pick up partial degraders or these synergistic effects in that level or tier of testing above the primary screening.

JOHNSON: Since you must respond to the ultimate jury or stockholders, this is a very expensive testing program you have outlined, could you estimate the cost from the beginning to the end where you would market a product?

GLEDHILL: We have one compound that has gone through this scheme, a detergent builder. It has gone through the confirmative level, and the cost is between one and two million dollars. This is why we need some decision on it. What has to be tested and how far do we test it?

RAYMOND: And I might remark that it's not the company that is paying for it. It is you.

BOLLAG: To what extent does your program correspond to current EPA regulations and to what extent is this your company's own initiative? This is an extensive program. Is there a possibility of comparison to other companies?

GLEDHILL: I don't want you to assume this is our process or used just by Monsanto. This is something that is being developed within various groups, especially the ASTM, who are considering methodology.

BOLLAG: To what extent does your program correspond to the requirements of EPA right now?

GLEDHILL: With the chemicals we are making now, we are not required to do testing, but we will be. As the rules come out we are required to follow them.

SOMERVILLE: Do you ever get any disparity between the results in the three screening procedures?

GLEDHILL: Yes, for example, when screening some compounds in activated sludge, we have gotten no degradation. Whereas, if we assess them in river water we can find sometimes that they do degrade.

SOMERVILLE: You are working at quite low concentrations, and this presumably means you have a specific analytical method for the compound. Would it not be acceptable to work at higher concentrations and use a generally applicable method like total organic carbon? Would this not reduce the cost of the screening procedure?

GLEDHILL: In the detergent and phosphate division of Monsanto, our screening procedure is based on total dissolved organic carbon. All of our products are simply water-soluble when added to a sterile activated sludge unit and they don't usually adsorb, so you can follow carbon disappearance. Other groups have products that do absorb, they have to look for a chemical on the sludge. That necessitates development of analytical procedures.

IVANOVICI: What range of organisms do you use for your toxicity studies? What criteria do you use to make the selection of these organisms?

GLEDHILL: For the aquatic toxicity studies, basically we are using algae, invertebrates and fish. There are various groups working on these organisms, standardizing methodology in this area. Essentially we use three types of algae, *Daphnia magna* as a freshwater invertebrate, and several fish including trout as a coldwater fish and the fathead minnow.

IVANOVICI: What sort of criteria do you use for actually assessing the effects on the organism? You mentioned LC_{50} as the final criterion, however, a lot of chronic effects would be overlooked although they nonetheless have severe long-term effects on the organisms. I am curious to see what sorts of other methods are being used.

GLEDHILL: Sure, there are very detailed methods to look at chronic toxicity, mostly standardized by ASTM groups. There isn't any methodology, that I know of, standardized for toxicity to microbial species. That is one thing that should be addressed here.

IVANOVICI: What, precisely, are these methods? O_2 uptake or changes in behavior?

GLEDHILL: No, they are just exposures looking for toxicity in the predictive level of testing. We are trying to establish a chronic or long-term toxicity at low-level concentrations.

IVANOVICI; I guess we could argue some more about how you actually evaluate these effects.

GLEDHILL: Well, I don't want to get into actual methods; they are detailed, published, and available. I am not an aquatic toxicologist, so a lot of them I am not capable of defining. I think the important consideration in the aquatic toxicity is trying to establish what they call an MATC value. It's maximum allowable toxicant concentration, and this is what we feel is the maximum amount of the compound that could be in an environment that does not have an effect on an organism.

PRITCHARD: Dr. Lee, I worry that in an evaluation like this where you are deriving a half life, whether CO_2 production is perhaps only part of the full story. The reason I say this is based on some of our studies. We seem to get a relatively small amount of CO_2 evolution from some compounds and yet, if you analyze the system more carefully you find degradation products in the water. You can make the assumption that these products will probably be eventually converted to CO_2, but due to the natural limitation of your system or the environment, you just don't see that much conversion. Therefore, CO_2 plus information on degradation products may be more indicative of the extent of degradation.

LEE: Well, I agree and I disagree. I said that is my limitation; we are not looking at the intermediates. We have tried to work with hydrocarbons and looked at compounds which are water-soluble, such as phenolic compounds. In these experiments we extracted the parent compound with something like chloroform, then acidified and then get the phenolic compounds into chloroform and count those. We see a 1:1 relationship between the phenolic compounds and CO_2. However, I am not saying that in some cases there isn't a buildup of metabolites. I feel that at least with hydrocarbons once the hydroxylation occurs the phenols are metabolized to CO_2, the rate limiting step being hydroxylation. Now with other compounds it may be something else.

PRITCHARD: If we are using a mathematical model to predict exposure concentration, the degradation rate we put into that model may be considerably off if CO_2 production is used for the rate determination.

LEE: Well, I very deliberately didn't mention the word model. I am not asking anyone to put this into a model.

PRITCHARD: Your first order rate constant that you obtain may be a reflection of the closed nature of the system rather than the ecology of the water sample.

LEE: I didn't present the data, but we have done experiments with large sediment trays and calculated rate constants. They are not that far apart from our bottle experiments. We feel that our bottle experiments do have validity, although the rate constants may not be totally realistic. I am not saying that there are not problems with bottle experiments but I think it is a first approximation. I'm not going to say any more on that.

FLOODGATE: I am a little disturbed that you continue to use this wretched measurement of half life which implies an exponential decay. Clearly your curves didn't show an exponential decay.

LEE: Why do you say that?

FLOODGATE: Well, they just didn't look like it from here.

LEE: It is a first order rate equation, that's all. The correlation coefficients are 0.98 to 0.99, generally. It depends on the compound. Some compounds fit it better than others, but it's not like glucose. Is that what you are getting at? It's not the same kind of degradation rates.

FLOODGATE: Wouldn't it be better to use turnover time or something of this sort, rather than half-life?

LEE: Well, I tried to do that. You're in microbiology, I am not a microbiologist. Turnover time doesn't mean anything to most people except to microbiologists. I think it's unique to that profession as far as I can see. But half-life means something to apparently everybody. I read all of Dr. Wright's papers and all the Michelis-Menten kinetics stuff. That stuff doesn't work at all. This is not Michelis-Menten kinetics here, it just doesn't square with that.

FLOODGATE: It's first order reaction, right?

LEE: That's right, it's not a linear equation. We don't get saturation kinetics.

FLOODGATE: You should really consider, though, very carefully. I think you should give both values, give turnover time and half-life to satisfy the microgists and wherever you happen to be.

LASKOWSKI: Just a comment on your 2,4,5-T data, we have run soil studies with 2,4,5-T, and our half-lives at 25°C have been in the range of 30-60 days.

LEE: That's not far off.

LASKOWSKI: No, I think that is very good.

LEE: Oh, you're agreeing. Okay.

LASKOWSKI: Just one additional comment on the use of $^{14}CO_2$ data. When that is used, I think in most cases it is an underestimation of decomposition, and that's the way to go.

RAYMOND: You agree, right?

LEE: I'm not saying anything.

ROGERS: Dr. Eggert, first of all let me compliment you on what seems to me to be quality work in microcosm work at this stage. My question is, what kind of indices do you use to characterize your aquatic system? I believe your terminology was relatively clean and minimally impact?

EGGERT: I use those words gingerly, but I guess compared to Lake of the Ozarks and the Mississippi River it seems to me to be a clean lake.

ROGERS: The reason I bring this up is that I believe very strongly in microcosm studies. It is very important to characterize the parent system such that your final result can be fairly weighed and interpreted.

EGGERT: I agree, this has been pointed out not only in our microcosm task group but also through discussions here. One of the things we will be undertaking is to get more information on the effect of bringing the water and sediment from the lake into the laboratory, and another thing is to try to characterize what is actually taking place during this transplantation.

GIDDINGS: You showed us how replicable your microcosms were, and that corresponds with our experience with similar systems. However, I wonder if you have any insights on how repeatable those experiments are? If you did the same thing six months later, would you get similar degradation rates?

EGGERT: My expectation would be that if we went out to the lake actually six months after we took the initial experiment's water samples and tried to reconstruct the whole thing, at least on a rate basis, I don't think it would be the same. That's my impression. In terms of LAS biodegradation and formation of various intermediates, I think the pattern would probably be the same. I don't expect that much variation.

GIDDINGS: You are still going on intuition the way we are?

EGGERT: Oh, yes, sure, no data on that.

RAYMOND: This microcosm is not a continuous flowing system?

EGGERT: No, this is a static system.

RAYMOND: I am sure you have been discussing this in your microcosm task group, but you really have no way to put nutrients into it during a long period of time that would be available to a lake, do you? It is just sitting there in that static conditions.

EGGERT: Yes, that is a problem. In my estimation, you don't have the inflow of freshwater that you might get with continuous flow systems to make sure that you have the nutrients. At one point I actually considered adding nutrients but decided against it. That was based on the fact that looking at what was going on physically from a qualitative point of view, the community really does seem to stabilize. I think that Jeffrey Giddings has noticed that, too. The sediment stratified and all kinds of invertebrates and plants grew, and it just appears healthy and ongoing. Now whether it is or not is another question.

SOMERVILLE: I believe that Dr. Cane of the University of Canterbury has microorganisms that will attack LAS in the ring before they attack the outside constituents. He also has organisms that will first attack the sulfonate substituents; so it would not be necessary to invoke a C-1 removal.

EGGERT: Yes, I was aware of that.

RAYMOND: But you've really seen no evidence for having those types of intermediates?

EGGERT: No, and another thing to point out is that the HPLC method is basically specific for the sulfo- phenyl-carboxylates of varying chain lengths.

What happens to the short-chain intermediates, once they're blown apart, we couldn't follow with this analytical procedure.

CRISTOL: This is related to the question that I think Dr. Raymond asked. You actually have enough data on the disappearance of your starting material and the appearance of carboxylic acids as intermediates to determine how your system is changing, by following rates by a steady-state treatment. Have you analyzed your data in this fashion?

EGGERT: Yes, there are quantitative data, extracted from the chromatographs, but I chose not to present them here. The extraction of rate data from a microcosm, at least from this microcosm, I felt was premature based on the fact that the system is still relatively complex. We didn't do enough experimental work to make a competent estimation of rates at this time.

RAYMOND: I think we'll open this now to questions for all four of the speakers of the morning. And we are going to give Dr. Cooney an opportunity to ask his question.

COONEY: Dr. Plimmer's discussion of UV-mediated photooxidation prompts me to make a comment about a microbial ecology phenomenon that I think most microbiologists ignore. If we are working in a niche that has visible light and oxygen, there will usually be extensive killing of microorganisms. We can see six logs of killing in a bright sunlit situation in estuarine water. I think this is important for those who are sampling slicks and for those who are sampling at different times of the day. I think we ignore it.

BRINK: I have a question for Bill Gledhill. The various complexities of analysis and material balance determinations has brought suggestions by some people that, even at the screening level, there should be a requirement for labelled compounds. What would your reaction be to that kind of requirement?

GLEDHILL: First of all, there are numerous types of compounds that can't be adequately labelled so this is a problem. There are also numerous types of new compounds that are produced in a few hundred pounds and maybe not used in any widespread use. It depends on volume/use/disposal characteristics as to whether you should do a labelled compound experiment. Most of the compounds that are produced in our area, because it is a large-volume product, we do have the labelled material synthesized. After the screening level studies, then we establish whether we are further interested in the compound.

MACALADY: I have a question for Dr. Lee. In the winter/summer differences which you observed, are those strictly temperature differences, or are there other water parameter differences like seasonal salinity variations and so on? Do you think the effect was just from temperature?

LEE: Well, no, it's related obviously to temperature, but I think we've heard some talk here that there are fewer bacteria per milliliter of water in the winter than there are in the summer. Is that related to temperature or is there some other reason? In our area, for instance, there is less detritus in the water in the winter than there is in the summer. No, I don't think you can make a straightforward, strictly temperature-related phenomenon. In the colder time of year, there is less degradation, but I certainly wouldn't say it is necessarily totally related just to temperature.

MACALADY: I am particularly interested in salinity. Were there salinity variations on a seasonal basis or not?

LEE: Yes, but it's not a predictable kind of thing. The salinity average in our area is around 24 ppt and it can go down to 14 ppt if it rains for three weeks, which it does fairly often. And it can rise a bit, too, with the tide bringing in highly saline water. We didn't see a difference between high and low tide degradation rates. So I just cannot really answer that. There may be some changes but we haven't seen them.

GIDDINGS: I have a question for Dr. Lee. Were the differences between winter and summer degradation rates for those various compounds consistent with regard to ranking at the different seasons?

LEE: They are very consistent. We can't see any degradation at all, in the winter for hexachlorophene and DEE; trichlorophenol is quite a bit lower. I can say that all of those compounds showed the same relative degradation rate in the winter. For example, the degradation rate for chlorophenol is much higher than for trichlorophenol. I assume that is the question.

COLWELL: I think there is a species basis for your observations. We have observed in Chesapeake Bay, in general, that in the summer months, the predominant species are *Vibrio* and, in the winter *Pseudomonas* and *Acinetobacter*. There is a seasonality demonstrated by species of these bacterial genera. I think this may very well be associated with the phenomenon you have recorded. In your offshore samples, did you take sediment cores or were you working only with water samples?

LEE: We haven't done any sediment work with offshore water. It's been kind of rough this year, and I get seasick real easy.

COLWELL: A lot of us get seasick. We just keep on working on one side of the research vessel and move to the other side when necessary.

LEE: I will remember that.

COLWELL: I think you will find that, in sediment there is a great potential for degradation. In the kinds of experiments you describe, you ought to speak in terms of degradative potential, not half-life. You see, even though you've got large populations in the sediment yielding faster rates of degradation, under natural conditions you will not have optimum exposure of the microorganisms to the material to be degraded. Nor will there be the concentration of oxygen and nutrients well circulated as in a flask in the laboratory. Thus, the results observed with sediment are a function of optimization of conditions, as well as larger populations.

LEE: I agree with you. I think you coined that term potential, probably with your hydrocarbon work. I certainly agree--this is potential. Although we have done experiments where we adapt the population until we get an enrichment.

Using these organisms we have regular Michelis-Menten kinetics and things fall into place. It's a pure culture. I agree, potential, I think, is a much better term to use for this.

STEEN: We often talk of rates which simply are not extractable quantities to what is happening in the environment. At the various concentrations we use to try to extrapolate with the right mathematical manipulations, both Michelis-Menten kinetics of Monod kinetics become first-order functions. In most cases we're exceeding the solubility and the K_s or saturation constants for Michelis-Menten kinetics to be obeyed. So I am very leery of trying to extrapolate rate data to environmental situations when we all know the extreme differences in rates that we get under different conditions, different population levels. I think we can get an evaluation or definition of rate often mixed with rate constants, and they are two entirely different properties of a system. Really, the only valid extractable quantity for predicting what is happening in a system is a rate constant. In response to Dr. Colwell's point, a lot of the natural water systems we've looked at in terms of population levels, we find that at concentrations slightly below solubility, the population levels seem to reach about the same order of magnitude in almost all natural water bodies. Of course, considering your point that potential is greater within the sediment, that is true, only if we understand the partitioning responses of compounds that enter into that water system. What we have been able to show so far within an aqueous phase is a second-order response of population to concentrations within most natural freshwater bodies. There is a consequent decrease in the concentration of the compound as a function of time, there is also a simultaneous increase in the population levels if that compound is used as a sole carbon source. Our experimental design system relative to the compound we use is a very, very important point. If you take a compound that has a partition coefficient of maybe 80, put 10 grams of compound into the system, we might only get 15% of the compound adsorbed. If we increase the sediment to water ratio a hundred-fold, we get 90% of it absorbed even at a very low partition coefficient. So what we measure as aqueous phase rate or rate constant is much different depending upon how much compound is absorbed.

RAYMOND: Anybody like to comment?

GLEDHILL: We considered this aspect in our methodology section. We came to the conclusion that it's important in testing not only to look at the compound of interest but also at a range of standard compounds so that comparisons can be made.

RAYMOND: Dr. Colwell, I would like to get a clarification on something I expect I heard you say. Maybe I misinterpreted it. Did you say that in Dr. Lee's system, one would have a better system because it was more agitated than in the natural environment?

COLWELL: I was implying there are optimum conditions created in a laboratory flask where sediment is added to a growth medium. Dispersion of the oil amongst particulates is optimized. Bacteria adhere to particulates and bacteria in the interstitial fluid are exposed to the substrate to be degraded. Greater exposure to the substrate, plus the added nutrients, and the larger populations create a system that is optimized. The average count of total viable bacteria for sediment populations is usually in the order of 10^6-10^8/g. In a water sample, only 10^2-10^4/ml bacteria may be found. Just in terms of numbers, the probability of having a biodegrading organism present is greater when the numbers of bacteria are higher, i.e., conditions are optimized, in the case of sediment samples.

RAYMOND: My argument is that I would visualize the natural system to have a much better chance of getting the nutrients, exposure, everything, because I think his systems were really stationary.

COLWELL: Not when comparing water and sediment, as in this case. Water samples yielded a longer residence time (half-life).

RAYMOND: My concern is that when we put these artificial systems together, we get this big bloom that, in a sense, takes away nutrient availability to further adapt a system to get biodegradation. I think you are limiting yourself on biodegradation possibility in these systems.

COLWELL: Yes, in closed systems.

LEE: Well, I think that in some cases sediments are being dispersed, at least with a six-foot tide. Have you ever seen our area? You can't see your nose in front of you most of the time. It's stirred up constantly so the sediment/water is constantly being mixed. I agree, if you want to talk about subsurface degradation, you've got a whole new set of problems to be worrying about. But that top centimeter, as far as I can make out, is being mixed constantly.

COLWELL: Yes, but you obviously have differences between the water and the sediment sample. Have you done any plate counts, biomass determinations, epifluoresence measurements, or other measurements to determine the difference in numbers?

LEE: No.

RAYMOND: All right, is anyone else going to question our methodology that we have had demonstrated here this morning?

PRITCHARD: Dr. Gledhill, I would like to address this question to you, to provoke discussion regarding the initial screening test you proposed. Do you feel perhaps that we could eliminate the activated sludge test? Perhaps we could simply do a river die-away test in which sediment was included.

GLEDHILL: If we want to try to develop one standardized test or something like that, it might be good to use something with soil as an inoculum. Essentially, that is what river water is; it receives water and soil from the banks of the river. Probably a lot of the organisms in a sewage plant do originate from soil. I think soil is more complete in microbial populations than an activated sludge plant. It might be good as a screening procedure to use a river water biodegradation test.

RAYMOND: That makes all of us soil microbiologists feel very good.

HOLLIS: Do we have enough information developed now to determine whether we use oxygen consumption or CO_2 evolution? I would like to have some comments from those folks that can lay out the protocol under which conditions you use oxygen consumption or CO_2 evolution.

FLOODGATE: I think it is simply a question of the system you are interested in. If you are interested in the pond or a river where there isn't a great deal of buffering action, then you can use CO_2 evolution and it's fine. But the sea is a highly buffered system; this is one point that perhaps we should remember more than we do. We all should remember from our first speaker this morning that pH changes are important. Well, pH changes in the sea are much more difficult to make because there is a strong buffer action due to the dissolved CO_2 in the water giving us a carbonate/bicarbonate system. Therefore, if you are going to measure CO_2 evolved of any particular experiment, you are up against a real problem. You are not quite certain whether the CO_2 has come from the degrading substrate or from some of the buffer in the system already. So I think a simple answer to the question is, if you are using a marine system, you must think very carefully about the CO_2 method because you may be disturbing the buffering system and changing the environment. If you're working in a pond or river, you have no problem.

KAUFMAN: I feel that oxygen consumption, CO_2 evolution, in themselves, are not an adequate indication of biodegradation. This is one of the problems with our literature today. There is a lot of information based on a single observation of O_2 consumption increased over control; therefore, it is biodegraded. In my own work, I have finally come to the conclusion that the adapted bug in one hand and the metabolite in the other hand is the only real safe criteria for metabolism. Now that is not to say these are not useful indicators. But as the sole criteria, I think they are totally inadequate. I am familiar with some literature in which people in ground disposal programs have put as high as 5 tons of 2,4,5-T into the soil and measured total CO_2 evolution. Everything in excess of control was calculated back as coming from the parent material. You're putting in a terrifically high amount; we don't know what the toxicology is to a soil population. These chemicals when put into the soil at a rate like that can cause some desorption of compounds which may also be a readily oxidizable or a more readily oxidizable substrate for the organisms. In that particular case, after arguing with the people involved in it, they finally went back and did the chemical extractions. Rather than finding that 2,4,5-T at 5 tons per acre was 80% degraded in 56 days, they found 90% was still there.

RAYMOND: Yes, I think we can all cite examples of where it is extremely dangerous to use just one or the other method solely. The same way as numbers, for many years, the adding of hydrocarbons to soils or water would exhibit great increases in numbers. This was used as an indication of biodegradation; when in fact, there was no biodegradation taking place because there is a killing of worms, nematodes, and things like this at the concentrations they were using. This gave a great increase in numbers so it had nothing to do with biodegradation of the parent compound.

FLOODGATE: Can I just tell a story here about what happened to us in one of those pitfalls that you do find using oxygen uptake? We had an experiment in which we were running seawater through a tube coated on the inner side with oil. The idea was to have a moving body of water in a fixed oil surface. Now, we noted we were getting very good oxygen uptake. We were cheering like mad, saying the "bugs" are at it, and it is being destroyed. But we noticed, too, that the "bugs" seemed to go to sleep at night because every time the lights went out, the oxygen consumption went down. There was a very close correlation between the amount of light and the oxidation rate. In point of fact, this was purely a photolytic mechanism, taking up oxygen. The "bugs" hadn't yet started. After about a week, the photolytic action decayed, and the bacteria took over. So you do have these kinds of problems. The question is, what are you going to do? You have to get some kind of answer, and you can't always do the real one, which is to take the substrate and show that it has changed chemically. This is really the only way you can show it has biodegraded. In this particular case, this would have meant ruining the experiment which we wanted to run for another six to twelve months. So you really have to make some sort of compromise. You have to know the difficulties. But you still have to make some kind of attempt to get an answer depending on what your experiment is trying to show in the end.

TIEDJE: My comment relates to company size. I think virtually all environmental toxicology problems that we have had in Michigan has been due to small companies. In some cases, the compounds were first evaluated by large companies, but considered too risky. Then some small company, which puts no dollars into quality control or environmental testing, and has no financial reservoir to go back and collect money from in the end, produces these chemicals and gets into some serious accidents. On the other hand, we have very elaborate schemes such as Monsanto's that cost about two million dollars. In terms of the public need, this might be overdoing it, compared to the risk. Going back to the comment about the small companies, obviously if an expensive two million dollar protocol is what's in mind, all small companies are out of business. I am not sure that that's going to be a politically acceptable alternative, but it may be the correct answer.

PERSISTENCE AND EXTRAPOLATION

Chairperson, James Tiedje

PREDICTION FROM LABORATORY STUDIES OF BIODEGRADATION OF POLLUTANTS IN "NATURAL" ENVIRONMENTS

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ABSTRACT

The ultimate fate of pollutants in the environment is the critical factor in judging whether a material or compound is an ecological threat and whether it must be subjected to stringent regulatory control. The susceptibility to microbial biotransformation of specific compounds, identification of intermediates that are formed, kinetics of the biologically mediated reactions and final end products are determined in the laboratory. The compound is thus characterized and its established behavior pattern can be used in consideration of natural ecological impacts. Studies in the environment often demonstrate that the behavior pattern in the field varies from that predicted by the laboratory characterization. This may be due to the failure to include environmental considerations as part of the protocol for laboratory investigations. It is the environment that actually controls the biotransformation process and has a greater influence on the process than the microorganisms per se. Interactions between the environment and compound (substrate) also may be neglected. Examples are given to illustrate these points and specific suggestions presented for factoring environmental aspects into the transposition process to provide a more reliable base for prediction from laboratory data.

The ultimate fate of pollutants in the environment is a critical factor in judging whether a material or compound is an ecological threat and whether it must be subjected to stringent regulatory control. The susceptibility of specific compounds to microbial biotransformation, identification of intermediated reactions, and final end products, are determined in the laboratory. The compounds are thus characterized and it is presumed that their now established behavior pattern can be used in consideration of ecological impacts in natural environments. Follow-up studies in the environment often demonstrate

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that the behavior pattern in the field varies from that predicted by the laboratory characterization. This discrepancy is particularly serious when, in expectation of complete biodegradability, a compound and/or its intermediates are found to persist in the environment. A number of reasons going back to experimental details can be advanced to explain the inability to transpose or, more accurately, to predict occurrences in the field from laboratory-generated data. Specific points can be raised regarding the nature of the microorganisms involved, the accuracy of the suggested biotransformation pathways, the nutrients available, etc. These, although valid, do not address themselves to the issue. What is most pertinent is the orientation taken in approaching the problem rather than specifics. When one is concerned with the question of biodegradation in the environment it must be understood that the key controlling feature is the environment, which includes the substrate. This puts the microorganism in a secondary role under this specific circumstance. While the driving mechanism is, of course, the microorganism, the controlling and limiting mechanisms are the environment and substrate.

To assist in more accurate transposition from laboratory to native environment, laboratory studies should be approached from the point of view of environment and substrate rather than from consideration of the microbial process alone as being the most important factor. This does not mean that studies of microorganism/substrate interactions are insignificant. One must know if a compound is readily, incompletely biotransformable or even recalcitrant. What is questioned is the assumption that if microorganisms can be shown to transform a given substrate then such transformation occurs, in time, under any circumstance.

The function of the environment must be understood since laboratory protocol may include variables of temperature, nutrients, pH, oxygen tension, microorganisms, substrates, etc., that these are environmental considerations and the matter is being belabored. Not so. It is a matter of definition and point of reference and has a real bearing on confidence in forecasting the probability of events. This has been discussed previously specific illustrative examples (7). I shall present some examples from this earlier publication as they are pertinent to this workshop and shall elaborate the discussion for a class of compounds to include some additional and very recent reported findings that present a rather strong case for the point of view I am advocating. The following excerpt (7) serves as a general example to illustrate the necessity of an environmental focus:

"If we concede that microorganisms of one sort or another will unerringly single out susceptible substrates and that these organisms are ubiquitous, the course of biodegradation is governed primarily by the environment in which the organisms and substrates find themselves. The ability of the organisms to attack a susceptible substrate is controlled directly by the living conditions in which it finds itself. This is an obvious truism that is unfortunately rarely accorded the weight that it deserves in predicting behavior of a material based on laboratory studies. This can be illustrated if one considers the fate of crude oil in a marine environment. Literature is replete with data to show that certain hydrocarbons can be biotransformed by microorganisms and that these components in crude oils may be metabolized. As a result, proposals have been made to seed oil spills with microorganisms in mixtures or with a single genetically altered bacterial species. These recommendations cannot be applied to the field situation because the natural environment precludes this. The conclusion that crude oil pollution in the marine environment can be abated through microbial means does not give proper weighting in the ecosystem to representative conditions approaching natural environments. When we have thousands of barrels of crude released into the open ocean, the combined environmental effects of low temperatures, lack of nutrients, high pressure at ocean depths, and variable levels of oxygen make it highly improbable that such volumes of oil either on or below the surface will be biodegraded even if all of the oil was potentially biodegradable, which it is not. Further, the assumption is made that the organisms of interest will persist in the environment which is doubtful, and that the organisms, substrates, and even added nutrients are confined in close contact with one another as though they were present in a fermentor. The latter assumption certainly may not be borne out when the action of wind, wave, and current disperses and mixes the system."

That this quotation is still timely is illustrated in a recent newspaper article (10) describing the 1.3 million barrel crude oil spill off the coast of Brittany in France from the wreck of the tanker *Amoco Cadiz*. The article notes that among the entrepreneurs on the scene offering help in cleaning up the oil is a businessman who has "oil-eating bacteria" for sale. And it is just such a situation that I had in mind when I stated earlier that definition of the environment in a laboratory context differs from the definition of environment in nature. They are obviously not the same. The frame of reference in the first instance is the microorganism and in the second, the environment and substrate. The gentleman selling his bacteria prefers the first situation and, in my opinion, cannot succeed in his endeavors because he refuses to accept a different valid definition and circumstance.

In adopting the position of assessing the situation from the environmental rather than the biological point of view, we consider chemical and physical interactions between substrate and environment. This is often omitted in laboratory studies. In our preoccupation with unravelling the course of microbial activity we may forget that we are dealing with compounds that can be affected by non-biological stresses and present altered substrates that may behave quite differently from environmentally unstressed compounds. Physical, chemical and photochemical phenomena play a role in combining or disrupting molecules, altering the relative proportion of mixtures of compounds, volatilizing or leaching components of mixtures or removing materials from solution or suspension through adsorption or encapsulation. If one considers the biodegradation of trinitrotoluene (TNT) isomers one must understand that under the influence of light so-called "pink water" may be formed which is a complex mixture that is a different chemical entity from TNT itself. It is this mixture that the microorganisms are exposed to if munition plant TNT effluents find their way into streams.

The necessity for consideration of non-biological parameters is documented in the recent paper by Herbes and Schwall (4) who studied the microbial transformation of polycyclic aromatic hydrocarbons (PAH) in pristine and petroleum-contaminated sediments. Their closing paragraph is exactly to the point. "The present work indicates that microbial transformation in sediment may be quite important in influencing the environmental transport of two- and three-ring PAH, but is much less important for the larger PAH compounds. The microbial rate data must be considered in conjunction with rates of other environmental transformation processes, including photolysis, bioaccumulation and sorption/sedimentation to assess adequately the transport of PAH in natural waters."

I should now like to confine my remarks to a discussion of a specific class of compound that will serve as an example of the point I am making. Again part of the discussion is taken from a previous paper (7), with some elaboration for this workshop.

"Di-2-ethyl-hexyl phthalate (DEHP), also commonly designated as di-isooctyl phthalate (DOP), when used as a plasticizer in polyvinyl chloride (PVC) films is resistant to microbial degradation in soil burial and plate tests (8, 9). Stahl and Pessen (11) showed that laboratory-prepared DEHP would not support growth of *Aspergillus versicolor*, but commercial preparations supported slight growth of *Pseudomonas aeruginosa*. Berk et al. (1) showed that DOP supported growth of some of the fungi studied after prolonged incubation, and trioctyl phosphate, di-butyl phthalate (DBP), and di-octyl phthalate showed evidence of supporting growth, but the growth was not conclusive enough to class them as carbon sources. In practice, over many years, in a variety of uses, and under all sorts of situations favoring microbial growth, PVC films containing DEHP have not been degraded to the point where their functionality is compromised within their use lives. Recently, reports have been issued indicating that DBP and DEHP can be utilized by microorganisms. Johnson and Lulves (5), in a most extensive study using ^{14}C radio-labeled compounds assayed in a fresh-water hydrosoil system, found that 53% of the DBP was degraded aerobically within 24 h and 98% within 5 days. DEHP was degraded only 50% after 14 days. Under anaerobiosis, degradation of both esters was retarded."

There have been other papers including a series from Germany, the most recent being that of Engelhardt and Wallnöfer (2), dealing with the microbial metabolism of dialkyl phthalates. They traced the course of hydrolysis of these compounds to the corresponding monoesters and free phthalic acid. Phthalic acid was then metabolized via protocatechuic acid by 3,4-dioxygenative ring cleavage. Kurane et al. (6) isolated gram positive and negative bacteria and two strains of fungi from soil and waste water samples capable of assimilating DEHP and other phthalate esters. They stated that a strain of *Pseudomonas acidovorans* was able to completely mineralize phthalate esters.

The literature demonstrates clearly that the aforementioned compounds can be biotransformed, yet when we incorporate them into a vinyl film, they are not amenable to biotransformation and the formulated polymer is judged to be resistant to microbial degradation. Further, Giam et al. (3) have shown that DEHP and DBP have been detected in the open ocean environment to the extent that they, among others, document phthalate ester plasticizers as a new class of marine pollutants. In general, they found higher concentrations of DEHP in water, sediment, air and biota of coastal regions than in the open Gulf of Mexico or in the Mississippi delta with lower concentrations in the North Atlantic. Why, if these compounds can be metabolized do they accumulate in the marine environment, not only in anaerobic areas but also in habitats where one can assume dissolved oxygen to be present? Obviously, the environment plus the substrate, in the case of the formulated plastic, and the environment alone, in the instance of the free plasticizer itself, influence the course of action; therefore, we find persistence instead of biodegradability.

If we concede to the environment its proper niche, what steps do we take to factor into laboratory protocols environmental parameters that may ease the task of transposition? I suggest that a given environment must be as thoroughly characterized as possible, and non-biological interaction between environment and substrate be considered in addition to the simple listing of such parameters as temperature, pH, dissolved oxygen, etc. When one speaks of a sediment, what are the exact microenvironments that actually exist in that sediment, what is its chemical nature and its physical state? Further, are there currents that shift the sediment, is macro debris accumulating that contributes to that environment, are the compounds moving or fixed, is light a factor, etc.? What is the chemistry of the substrate in that specific environment, is it reactive, can the substrates polymerize and undergo gross conformational reorganization when they actually are physically so complex at the micro- and macromolecular level that extracellular enzymes, for example, cannot reach susceptible linkages? What other compounds are present? If we have a mixture, then this must be considered rather than the single compound. Methods and instrumentation must be developed to monitor the salient natural microenvironments and these actual conditions and sequences used for development of the laboratory ecosystem. Obviously, it is impossible at present to characterize an environment in its entirety or to bring the total environment into the laboratory, nor may it be necessary. Judgments can be made to a greater degree than is now done, to select those factors from a given environment that may have major impact. Since even this is difficult, proper and judicious use of the computer to assess different circumstances comes into play as an aid in judging what factors must be included in the laboratory ecosystem environment.

In the time allotted to me I have not gone into great depth of discussion that a suggested change in outlook and philosophy deserves. I trust that my presentation serves as the base for an exchange and elaboration of comment and thought. An awareness of the necessity for more significant consideration of the environment and the substrate is being evidenced during this workshop. I sense an uncertainty in knowing what must be done and how to do it, and I still find an emphasis on the microorganism in those situations where the emphasis must be elsewhere. When one shows concern over washout of a specific organisms from a mixture in a continuous flow model ecosystem, presumably representing a native situation, when concern should be with the change in the substrate under this situation, I think the point is made.

Finally, it is not scientifically valid to skew a situation by neglecting to give full and equal consideration to all elements involved. One must devote as much time, effort, and skill to the environment as it influences substrate/microorganisms interactions as has been devoted to microorganism/substrate interactions alone.

LITERATURE CITED

1. Berk, S., H. Ebert, and L. Teitell. 1957. Utilization of plasticizers and related compounds by fungi. Ind. Eng. Chem. 49:1115-1124.

2. Engelhardt, G., and P. R. Wallnöfer. 1978. Metabolism of di- and mono-n-butyl phthalate by soil microorganisms. Appl. Environ. Microbiol. 35:243-246.

3. Giam, C. S., H. S. Chan, G. S. Neff, and E. L. Atlas. 1978. Phthalate ester plasticizers: a new class of marine pollutant. Science 199:419-421.

4. Herbes, S. E., and L. R. Schwall. 1977. Microbial transformation of polycyclic hydrocarbons in pristine and petroleum-contaminated sediments. Appl. Environ. Microbiol. 35:306-315.

5. Johnson, B. T., and W. Lulves. 1975. Biodegradation of di-n-butyl phthalate and di-2-ethylhexyl phthalate in fresh water hydrosoil. J. Fish. Res. Bd. Can. 32:333-339.

6. Kurane, R., T. Suzuki, and Y. Takahara. 1977. Microbial degradation of phthalate esters. I. Isolation of microorganisms growing on phthalate esters and degradation of phthalate esters by *Pseudomonas acidovorans* 256-1. Agric. Biol. Chem. 41:2119-2123.

7. Kaplan, A. M. 1977. Microbial degradation of materials in laboratory and natural environments. Dev. Ind. Microbiol. 18:203-211.

8. Kaplan, A. M., M. Greenberger, and T. M. Wendt. 1970. Evaluation of biocides for treatment of polyvinyl chloride film. Polymer Eng. Sci. 10:241-246.

9. Klausemeier, R. E. 1972. Results of the second interlaboratory experiment on biodeterioration of plastics. Int. Biodeterior. Bull. 8:3-7.

10. Revzin, P. 1978. Spilled oil tide engulfs Brittany coast, washes up shipload of major questions. Wall Street Journal, March 27.

11. Stahl, W. H., and H. Pessen. 1953. The microbiological degradation of plasticizers. I. Growth on esters and alcohols. Appl. Microbiol. 1:30-35.

USE OF MICROCOSMS TO ASSESS MICROBIAL DEGRADATION PROCESSES AND SEDIMENT-WATER INTERACTIONS IN RESERVOIRS THAT DEVELOP ANAEROBIC HYPOLIMNIONS

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ABSTRACT

A study was conducted to assess the feasibility of using microcosms to investigate changes in biochemical, chemical, and microbial parameters occurring during development of anaerobic conditions in reservoirs. In all instances, rapid decreases in oxidation-reduction potential occurred during the first 24 h of incubation, strongly paralleled by depletion of dissolved oxygen in the same systems.

Examination of changes in concentrations of reduced soluble iron and manganese indicated that manganese reduction preceded that of iron by 3 to 5 d, being much more extensive than that of iron reduction. Manganese reduction was not observed at redox potentials above +100 mV; redox potentials well below 0 mV were apparently required for iron reduction to occur.

Examination of changes in forms and amounts of nitrogen with anaerobic incubation showed a decline in nitrate-nitrogen and an accumulation of ammonium nitrogen. Data suggested that nitrate depletion was largely due to denitrification while ammonium accumulation resulted principally from mineralization of organic nitrogen.

Changes in the pattern of organic acids and alcohols produced during periods of anaerobic incubation indicated a gradual shift from patterns closely resembling alcoholic and propionic fermentations to much more complex mixed fermentations. Acetate was present in greatest abundance, and its accumulation was considered in relation to the potential for methanogenesis.

Results strongly parallel findings of other investigators working with flooded soils and sediments. The only major disparity found was in the area of regulation of the degree of oxidation-reduction by inorganic electron acceptors. This disparity is discussed in relation to current theories on the nature of oxidation-reduction potential and the role of mixed oxidation-reduction potentials exerted by a mixture of organic and inorganic redox couples. It is concluded that microcosms represent a viable means of studying anaerobic processes in flooded soils and sediments, and with some care, may also be used to study such processes in reservoirs.

INTRODUCTION

Water flowing through impoundments can undergo several intensive changes in its biological, chemical, and physical properties. Some water quality properties, such as turbidity, may be enhanced by the temporary retention of water in a reservoir (16); other water quality parameters can be seriously degraded by this process (15). This situation is complicated by the fact that the hydrodynamic pattern of many large multi-purpose reservoirs is quite different from that of natural lakes (26). While natural lakes discharge from the surface, many reservoirs draw their discharges from deep hypolimnetic waters. Such withdrawals are often desirable in terms of meeting cold water temperature objectives for downstream fisheries. However, in recent years the Corps of Engineers (CE) has had several problems associated with the adverse environmental impacts of anoxic hypolimnetic waters accompanied by products of several CE reservoirs (Keeley et al., in press). Examples of undesirable materials that have been included in these releases are reduced forms of iron and manganese, hydrogen sulfide, and organic compounds having objectionable odors.

To best address the problem of preventing or ameliorating circumstances that lead to release of anoxic waters from reservoirs, a research program was initiated to: provide basic data on the causes of anaerobic conditions in reservoirs; develop predictive techniques for determining potential environmental impacts associated with development of new impoundments; and assess the effects of specific changes in operational strategy on the quality of waters released from existing impoundments.

We are using microcosms along with a mathematical ecological model and field studies of existing and proposed impoundments in our investigation of reservoir processes. While the use of microcosms admittedly has limitations in application of "real world" situations, it has been successfully applied by Jørgensen and Fenchel (7) and others to study microbial geochemical processes in marine and fresh water sediments. Moreover, Russian investigators have verified the utility of microcosms in forecasting the water quality of a cascade of reservoirs on the Dnieper River (11).

The present paper reports the results of our initial studies to assess the feasibility of using microcosms to investigate changes in biochemical, chemical, and microbial parameters that occur during development of anaerobic conditions in reservoirs.

MATERIALS AND METHODS

DECOMPOSITIONAL MEDIUM

To eliminate the variability encountered when using natural vegetative substrates, a defined anaerobic medium (DAM) containing known quantities of important plant constituents was formulated. This medium contained, per liter: 100 ml of basal fresh water medium (Table 1); cellulose powder, 1.0 g; xylan, 0.4 g; pectin, 0.3 g; Phytone^{TM*} (BioQuest), 0.2 g; cellobiose, 0.05 g; soluble starch, 0.05 g; $CaCO_3$, 1.5 g; final pH, 8.2.

TABLE 1. COMPOSITION OF BASAL FRESH-WATER MEDIUM USED IN THE STUDY OF ANAEROBIC DEGRADATION PROCESSES[a]

Component	Amount
$NaNo_3$	0.100 g
$CaCl_2 \cdot 2H_2O$	0.034 g
$MgSO_4 \cdot 7H_2O$	0.056 g
K_2HPO_4	0.106 g
KH_2PO_4	0.053 g
$NaHCO_3$	0.060 g
$Na_2SiO_3 \cdot 9H_2O$	0.028 g
H_3BO_3	0.011 g
Iron stock solution A[b]	1.0 ml
Iron stock solution B[c]	1.0 ml
Micronutrient stock solution[d]	1.0 ml
Trace organic stock solution[e]	1.0 ml
Glass distilled water	1000.0 ml

[a] Medium is normally used at one-tenth of full-strength, unless otherwise noted. Medium can be sterilized by membrane filtration.

[b] Contains 2.49 g of $FeSO_4 \cdot 7H_2O$ and 0.5 ml of H_2SO_4 per liter of glass distilled water.

[c] Prepared by reacting 31 g of KOH with 50 g of Ethylenediaminetetraacetic acid (EDTA) in 800 ml of glass distilled water. After solution has cooled, 1.58 g of $FeCl_3 \cdot 6H_2O$ is added, and the final volume adjusted to one liter with distilled water.

[d] Contains, in g per liter of glass-distilled water: $ZnSO_4 \cdot 7H_2O$, 9.0; $MnCl_2 \cdot 4H_2O$, 4.46; MoO_3, 0.80; $CuSO_4 \cdot 5H_2O$, 0.04; and $Co(NO_3)_2 \cdot 6H_2O$, 0.045.

[e] Prepared by dissolving 0.1 g of yeast extract in 100 ml of glass distilled water. A 10-ml portion of this is then diluted to one liter with glass distilled water to prepare the stock solution.

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*Use of trade name materials in this paper does not constitute endorsement by the U.S. Government.

To provide inorganic electron acceptors for the study of anaerobic decomposition processes, 0.1 g of each of one or more of the following substrates were added to each liter of DAM used: nitrate-nitrogen as KNO_3, manganese as an amorphous manganese dioxide, and iron as ferric oxyhydroxide. Preparation of the manganese dioxide and iron oxyhydroxide will be described elsewhere (Brannon, Gunnison, Butler, and Smith, USAE Report TR Y-78-11, in press).

INOCULA

An inoculum rich in aerobic microorganisms was prepared by adding enough DAM to 4.5 g of a fresh garden soil to bring the volume to the precalibrated 100 ml mark of a standard milk dilution bottle. This preparation was thoroughly shaken, and the resulting suspension was permitted to settle for 10 sec. At this time, the desired volume of inoculum was removed from the top 3 cm of suspension using a syringe fitted with a 21 gauge, 1-1/2" needle. Inoculation was accomplished by injection through a rubber septum on the culture vessel.

An inoculum rich in anaerobic microorganisms was prepared as follows. To 90 ml of DAM that had been purged for 10 min with oxygen-free nitrogen was added enough anaerobic lake sediment to bring the volume to 100 ml. This preparation was stoppered under nitrogen. The remainder of the treatment was the same as for the aeroflora, except that precautions were taken to maintain the anaerobic integrity of the inoculum.

CULTURE CONDITIONS

Development of anaerobic conditions was studied in 1-, 2-, and 7-liter reaction vessels that contained 650 ml, 1800 ml, and 6 liters of the decompositional medium, respectively. Vessels were either commercially available or were constructed according to the description of Patrick et al. (19); these units were normally equipped for continuous monitoring of dissolved oxygen, oxidation-reduction potential, pH, and temperature. After sealing, vessels were charged with air until the medium was saturated with dissolved oxygen. Aeration was then terminated and the headspace of each vessel was purged with nitrogen for 1 min to dispel gaseous oxygen in the atmosphere above the medium. At this point, stirring of the medium was initiated, and it was inoculated with 1.0 ml/liter of each of the anaerobic and aerobic inocula. To insure the survival of strict anaerobes, inoculation of the anaerobes was repeated 24 h after the initiation of the experiment. Incubation temperatures ranged from 23 to 30°C with agitation rates of 200 to 700 rpm.

Time 0 samples were taken immediately after completion of inoculation. The period of incubation varied from 10 to 42 d, depending upon the size of the vessel and the intensity of sampling. Additional samples were normally taken at 1, 3, 6, 10 or 12, 17, 23, 40, and 42 d of incubation.

MEASUREMENT OF OXIDATION-REDUCTION POTENTIAL

Oxidation-reduction potential was measured on the ± millivolt scale of a standard millivolt-pH meter. Electrodes for measurement of oxidation-reduction potential were prepared as described by Mann and Stolzy (10). The reference potential was supplied either by a standard calomel half-cell that was connected to the culture medium with a saturated potassium chloride-agar bridge or by a standard pH calomel reference electrode that was immersed directly into the culture medium.

SAMPLE COLLECTION AND PRESERVATION

To maintain the anaerobic integrity of the samples, all procedures were conducted under a nitrogen atmosphere. Samples to be analyzed for soluble nutrients or for organic acids and alcohols were cleared of particular matter by passage through a 0.45-μm membrane filter. Samples for iron and manganese were passed through a 0.10-μm filter. Previous investigators have shown that the use of a 0.10-μm filter will remove metals that are in a colloidal state (8) and we assumed that all metals that passed this filter were in a reduced, soluble state.

Samples for total or soluble nutrients or for organic acids and alcohols were preserved by freezing at -10°C. Metal samples were preserved by acidification with concentrated (11.6 *N*) HCl added at the rate of 0.2 ml of acid per 15 ml of sample. Microbial samples were processed within 2 h of collection.

SAMPLE ANALYSIS

Analysis for the various forms of nitrogen was performed with a Technicon Autoanalyzer (Technicon Instruments, Inc.), using the manufacturer's recommended procedures.

Samples for analysis of organic acid and alcohol products were concentrated ten-fold on a rotary evaporator at 55°C. Sample concentrates were then prepared and analyzed for volatile organic acids, alcohols, and non-volatile organic acids using the procedures for analysis of anaerobic cultures as described by Holdeman and Moore (6). Analysis of standards treated in the same manner indicated a 50-70% recovery of all volatile constituents within the sample concentrate.

RESULTS

CHANGE OF OXIDATION-REDUCTION POTENTIAL, DISSOLVED OXYGEN, AND pH DURING PROLONGED ANAEROBIC INCUBATION

Oxidation-reduction (redox or Eh) potential was measured during the incubation period as an indication of the intensity of reducing conditions in the culture media and also as an indication of whether or not the active reduction of one or more of the inorganic electron acceptors resulted in a poising of redox potential. In all instances, the most rapid decreases in redox potential occurred during the first 24 h of incubation (Fig. 1). For the first few hours, this rapid decline in redox potential was strongly paralleled by the depletion of dissolved oxygen in the system. The simultaneous measurement of dissolved oxygen showed that complete depeltion occurred in the full-strength

DAM system within 6 h after initiation. The one-tenth strength DAM system was depleted of dissolved oxygen within 12 h, while the one-hundredth strength DAM system took nearly 72 h for complete exhaustion of dissolved oxygen. The initial drop in redox potential occurred during the first 10 to 15 min after the microcosms had been sealed off. At this time, residual oxygen dissolved in the medium was undoubtedly exchanged with the gaseous nitrogen that had been added to the headspace of the vessel. In any event, the redox value in the presence of oxygen is of dubious value because of the unstabilizing effect of oxygen. As noted subsequently, organic redox couples also may have exerted an influence. Redox values for longer incubation periods changed little from those shown at 10 days. During the same 10-d incubation period, the pH values of the three DAM systems ranged from 8.2 to 6.1.

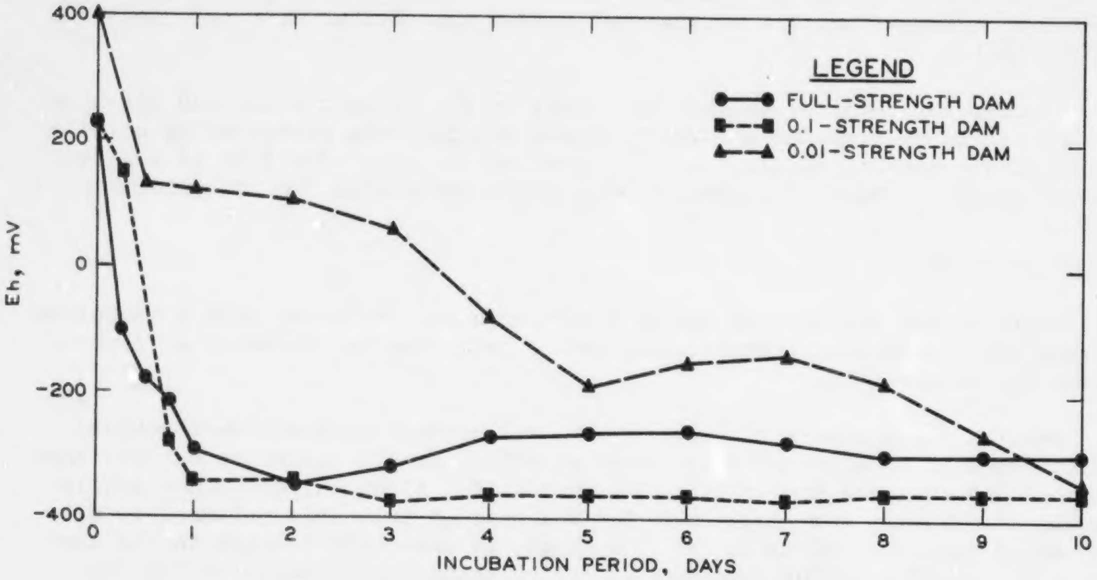

Figure 1. Changes in redox potential (Eh) during 10 days of incubation in media containing 3 different concentrations of organic matter.

REDUCTION OF MANGANESE AND IRON

To determine whether concentration of organic matter has any effect on the rate and amount of manganese and iron reduction, the concentrations of organic matter in DAM were varied from full strength to one-hundredth of full strength while the concentrations of oxidized manganese and iron were held constant. Changes in concentration of soluble manganese during 10 d of incubation in each of the three strengths of DAM are shown in Figure 2, while changes in soluble ferrous iron are depicted in Figure 3. The trends for manganese and for iron are similar in all media studied. Manganese reduction precedes that of iron by 3 to 5 d and is also much more extensive than that of iron reduction. The one-hundredth strength DAM culture showed minimal iron reduction when its incubation was allowed to proceed for an additional 10 d,

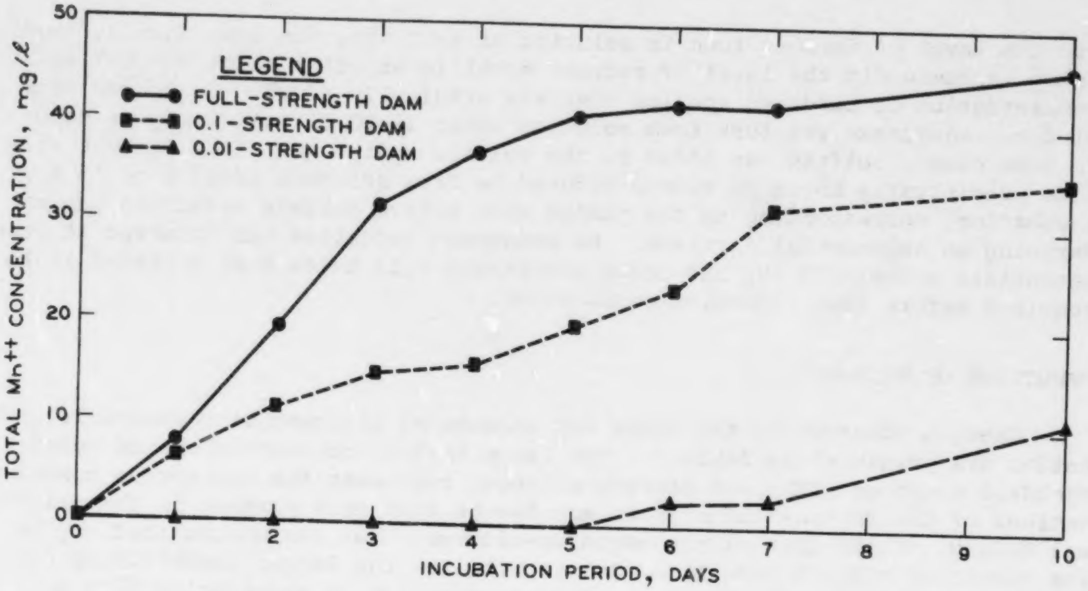

Figure 2. Changes in concentration of soluble reduced manganese during 10 d of incubation in media containing 3 different concentrations of organic matter.

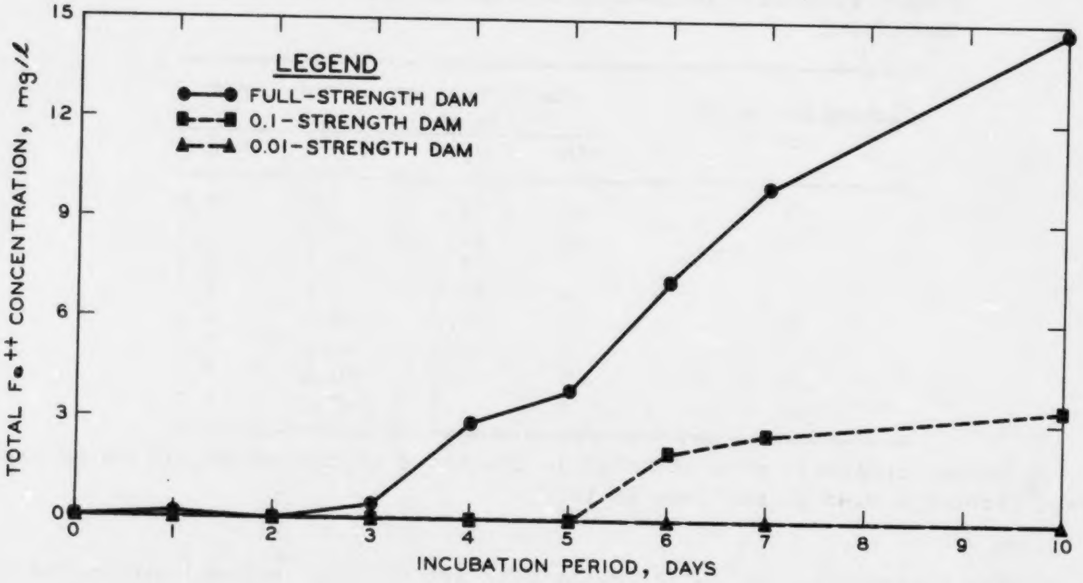

Figure 3. Changes in concentration of soluble reduced iron during 10 d of incubation in media containing 3 different concentrations of organic matter.

and the level of ferrous iron in solution at this time was less than 0.7 mg/l. In no instance did the level of reduced metal in solution reach the 100 mg/l concentration of oxidized species that was originally added. In longer term studies, manganese was lost from solution after 11-20 d, presumably as $MnCO_3$. In some cases, sulfate was added to the culture medium and black ferrous sulfide precipitates began to remove reduced Fe from solution after 6 to 15 d of incubation, corresponding to the period when active sulfate reduction was undergoing an exponential increase. No manganese reduction was observed at redox potentials above +100 mV, and redox potentials well below 0 mV appeared to be required before iron reduction could occur.

REDUCTION OF NITRATE

Changes observed in the forms and amounts of nitrogen with anaerobic incubation are presented in Table 2. The large initial concentrations of total Kjeldahl nitrogen (TKN) and nitrate-nitrogen represent the respective contributions of the Phytone and nitrate amendments that were present in the original medium. While some of the ammonium-nitrogen that was accumulated may be the result of nitrate reduction through nitrite, the larger contribution of ammonium-nitrogen probably results from deamination of amino acids (1); a source of this was present in Phytone. However, utilization is not indicated by a significant decrease in TKN that parallels an increase in ammonium-nitrogen.

TABLE 2. CHANGES IN CONCENTRATION OF THE VARIOUS FORMS OF SOLUBLE NITROGEN DURING PROLONGED INCUBATION OF FULL-STRENGTH DAM UNDER ANAEROBIC CONDITIONS

Incubation period (d)	Concentration of component in medium (mg/l)[a]			
	TKN	NO_3^--N	NO_2^--N	NH_4^+-N
0	60	84	1.5	2.5
1	21	32	3.2	3.2
2	5	2	<0.1	3.6
3	6	<0.5	<0.1	4.8
6	6	<0.5	<0.1	5.5
10	7	<0.5	<0.1	7.0
20	5	<0.5	<0.1	7.1
40	4	<0.5	<0.1	2.2

[a] Values indicated were obtained in filtrates of the medium following passage through a 0.45-μm membrane filter.

That denitrification is the major sink for nitrate in these microcosms is suggested by the failure to accumulate large amounts of nitrite, TKN, or ammonium-nitrogen in the culture. Moreover, additional experiments were performed wherein nitrates were added to manganese-iron reduction studies after 20 to 30 d of previous incubation. In this situation, an immediate rise in the redox potential was observed followed by a period during which the redox was found to be held above a level of approximately +100 mV. During this time, reduced manganese and iron were observed to be reoxidized by the added nitrate, and nitrate gradually disappeared from solution without the appearance of nitrite or ammonium. Once the supply of added nitrate had been depleted, the redox potential fell back to previous levels, and manganese and iron reduction were reinitiated (Brannon et al., USAE Report TR Y-78-11, in press). Additional support for denitrification as a sink for nitrate was demonstrated by the fact that inoculation of nitrate agar during periods of rapid nitrate depletion in the microcosms resulted in the presence of large populations of gas-producing microorganisms, a phenomenon indicative of denitrification (20). No such increase in the number of these organisms was observed in the absence of nitrate.

PATTERNS OF VOLATILE ORGANIC ACIDS AND ALCOHOLS

Changes in patterns of volatile organic acids produced during periods of anaerobic incubation are indicated in Table 3, while patterns for alcohols are presented in Table 4. These patterns, while not quantitatively definitive, do indicate a gradual shift in the pattern of fermentation products from those which closely resemble the "classical" alcoholic and propionic fermentations to a type which more closely resembles the more complex, mixed fermentations (25, 31). While our procedures did not account for evolution of methane gas, no large-scale evolution of gas was ever observed after 3-5 d of incubation, although concentrations of acetate, a normal substrate for methanogenesis (33, 36), continued to increase through the twenty-third day of incubation (Table 3).

TABLE 3. CHANGES IN PATTERNS OF VOLATILE ORGANIC ACIDS PRODUCED DURING ANAEROBIC DECOMPOSITION OF ORGANIC SUBSTRATES IN FULL-STRENGTH DAM

Incubation period (d)	Volatile fatty acid in culture filtrate[a]				
	Formic	Acetic	Propionic	Butyric	Valeric
1	0	++	0	0	0
3	0	++	+	0	0
6	0	++	++	0	0
12	+	+++	++	0	0
23	0	+++	++	+	+
42	0	0	++	0	0

[a] Values given are based on relative peak heights obtained by gas-liquid chromatographic analysis of concentrated culture filtrates. 0, acid not detected; +, peak height of 1-5% above baseline; ++, peak height of 6-50% above baseline; and +++, peak height of 51-100% above baseline.

TABLE 4. CHANGES IN PATTERMS OF ALCOHOLS PRODUCED DURING ANAEROBIC DECOMPOSITION OF ORGANIC SUBSTRATES IN FULL-STRENGTH DAM

Incubation period (d)	Alcohol in culture filtrate[a]			
	Ethanol	Propanol	Butanol	Pentanol
1	++	+++	0	0
3	++	++	0	0
6	++	++	0	0
12	++	++	+	0
23	+++	++	0	+
42	+++	+++	0	0

[a] Values given are based on relative peak heights obtained by gas-liquid chromatographic analysis of concentrated culture filtrates. 0, alcohol not detected; +, peak height of 1-5% above baseline; ++, peak height of 6-50% above baseline; and +++, peak height of 51-100% above baseline.

DISCUSSION

Our results suggest that the microorganisms and anaerobic processes involved in decomposition of organic matter and in reduction of nitrate, manganese, and iron in the microcosms studied are very similar to those occurring in flooded soils and sediments. When the supply of oxygen to a soil or sediment system containing decomposable organic matter is curtailed, profound changes occur in the composition of the indigenous microbial community and in the microbial processes involved. The succession of microorganisms includes first aerobes, and then facultative anaerobes where both populations achieve their maximum levels within the first 48 h after flooding (17); these are subsequently replaced by the strict anaerobic bacteria, to which O_2 is lethal once the supply of oxygen within the soil has been totally exhausted (27, 28).

The trends from the present data and those described in another study (Brannon and Gunnison, report in preparation) indicate that the successional sequence of the study microcosms is nearly identical to that of flooded soils and sediments, although some differences were noted. Dissolved oxygen was readily depleted within the first 24 h in both the full-strength and one-tenth strength DAM cultures. The populations of aerobes in full-strength DAM also peaked within the same time period, and the number of aerobes was well on the decline by 48 h (Brannon et al., USAE Report TR Y-78-11, in press). Takeda and Furusaka (30) observed that the number of facultative anaerobes always exceeded the total number of strict anaerobes in a paddy field soil. Our data for facultatively anaerobic organisms (Brannon et al., USAE Report TR Y-78-11, in press) indicate the same trend in that our total anaerobe populations were composed of both facultative and strict anaerobes, and the values of the facultative anaerobes above were always at least 90% of the value for the total anaerobes. Our results did not show any apparent differences in time for appearances of the peaks of the facultative anaerobe and the strict anaerobe populations; however, the facultative anaerobe population declined sharply after the first 4 d while the total anaerobe population began a gentle reduction in numbers only after 6 days of incubation. Moreover, some of our preliminary data indicate that populations of sulfate-reducing organisms do not achieve their maximum until 12 d after initiation of experimentation; this is significantly far behind the preceding populations and is in agreement with the data of Takai and Kamura (27) for populations of reducing microorganisms in a waterlogged paddy soil.

The chenical and electrochemical transformations that occur in flooded soils and sediments also have been well documented. Immediately after waterlogging, the redox potential declines due to the activities of aerobic and facultatively anaerobic bacteria (27). The drop in redox potential then stimulates the initiation of full activities of anaerobic and sulfate-reducing bacteria (27). Similar to results obtained by previous investigators working with flooded soils and sediments, our data show that nitrate disappears at the very beginning of the incubation period and that denitrification is probably the main sink for nitrate. Little reduction of Mn and almost none of Fe can occur until both oxygen and nitrate have been totally depleted (28, 29, 32).

The accumulation of organic acids and alcohols in a liquid culture medium is similar to that obtained for soils and sediments. Ponnamperuma (22) has summarized the transformation products of pyruvic acid that are present in anaerobic soils and cultures containing soils. All the products listed in Tables 3 and 4 are present in his list.

The major disparity between our results and those in the existing literature is in the area of regulation of the degree of oxidation-reduction by inorganic electron acceptors. Addition of large amounts of an oxidized inorganic electron acceptor to a submerged soil or sediment may stabilize the redox potential. Thus, the presence of NO_3^- in anaerobic systems has been found to stabilize the redox potential at values between +200 and +400 mV as long as NO_3^- is present (21, 23, 32, 34, 35). Moreover, addition of NO_3^- to anaerobic systems will either increase the redox potential or else retard a further decrease in redox potential (5). Similarly, the effects of Mn IV and Fe III reduction also retard the decrease in redox potential of flooded soils (14, 27). Our studies do not confirm a parallel retardation in the decrease in redox potential in our microcosms that could be attributed to the presence of Mn IV or Fe III. Generally, the redox potentials in our systems fall rapidly once the oxygen supply has been cut off, reach their lowest levels within 24-28 h, and return to levels that are slightly above the minimum values.

We hypothesize that the inability of Mn IV and Fe III to influence the degree of oxidation-reduction in our microcosms is due to the overriding influence of organic redox couples. Our hypothesis is suggested by the following results in conjunction with the findings of other workers: a) Addition of Mn IV or Fe III did not alter the rate or magnitude of decreases in oxidation-reduction potential observed in microcosms containing organic matter only. b) Observed Eo's (standard oxidation potential) calculated from Eh, pH, PCO_2 and Fe II and Mn II activities for Mn and Fe redox systems that can influence the oxidation-reduction potential diverged greatly from theoretical values. c) In our microcosms, a decline in oxidation-reduction potential could be retarded only by O_2, NO_3^-, or decreasing the organic matter to one-hundredth of its full strength (20 mg/l). d) Ponnamperuma et al. (24) found that the predicted regulation of Eh by Mn redox couples deviated least from theory in soils high in Mn IV and low in organic matter. Addition of organic matter hastened reduction and did not allow Mn redox systems to fully exert themselves. e) Addition of organic matter to anaerobic systems generally increases the reduction rate (22, 29). f) Flooded soils having low concentrations of reducible iron and manganese, along with large amounts of organic matter, show rapid declines in redox potential (18). g) Purging the microcosms with purified N_2 following the initial rapid drop in Eh produced no change in system Eh, ruling out the H_2-H^+ couple as a potential determining component. h) The demonstrated ability of reversible organic couples to exert electrode potentials (4). i) The ability of irreversible organic couples to exert electrode potentials via a "mediator" system (4, 9).

Even though iron and manganese redox systems were not solely responsible for the Eh observed in our microcosms, their redox couples were exerting an influence. Because of their electroactivd nature, Mn and Fe redox couples can act as "mediators" for irreversible redox systems (9). Iron and manganese redox couples may also contribute to the mixed potential that was probably regulating Eh in our systems. A mixed potential is essentially a weighted average of the potentials of all redox couples present, the weighting of each couple dependent upon its exchange current (2, 3). Patrick and Mikkelson (17) considered the redox potential of a submerged soil to be a mixed potential and felt that any shift in the redox potential signaled a shift in the oxidation-reduction equilibrium, usually involving changes in more than one system.

Our experiments have clearly demonstrated that microcosms represent a feasible tool for simulating anaerobic processes of flooded soils and sediments. The temptation exists to extrapolate the results obtained in these systems directly to anaerobic waters of reservoir hypolimnions; indeed, Ponnamperuma (22) has given thorough consideration to the movement of the boundary between the aerobic and anaerobic zones from lake sediments to above the mud surface, and into the hypolimnion. We feel that by this process the water of the hypolimnion becomes an extension of the sediment interstitial water, although the intensity of reduction and the reduction products observed may not be identical. Mortimer (12, 13) has observed that in eutrophic lakes which develop thermal stratification, the redox potential declines rapidly in the thermocline and may acquire, in the hypolimnion, values that are as low as those in the underlying reduced sediments. Thus, with some care, the results obtained with microcosms can probably be used to study and interpret processes that occur in anaerobic hypolimnions of reservoirs.

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LITERATURE CITED

1. Alexander, M. 1961. Introduction to soil microbiology. John Wiley and Sons, Inc., New York.

2. Bohn, H. L. 1968. Electromotive force of intert electrodes in soil suspensions. Soil Sci. Soc. Amer. Proc. 32:211-215.

3. Bohn, H. L. 1974. Redox potentials. Soil Sci. 112:39-45.

4. Clark, W. M. 1972. Oxidation-reduction potentials of organic systems. Robert E. Krieger Publishing Company, Huntington, New York.

5. Engler, R. M., and W. H. Patrick, Jr. 1974. Nitrate removal from floodwater overlying flooded soils and sediments. J. Environ. Qual. 3:409-413.

6. Holdeman, L. V., and W. E. C. Moore (eds.). 1973. Anaerobe laboratory manual, 2nd ed. VPI Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

7. Jørgensen, B. B., and T. Fenchel. 1974. The sulfur cycle of a marine sediment model system. Mar. Biol. 24:189-201.

8. Kennedy, V. C., G. W. Zellweger, and B. F. Jones. 1974. Filter pore-size effects on the analysis of Al, Fe, Mn, and Fe in water. Water Resources Res. 10:785-791.

9. Laitinen, H. A. 1960. Chemical analysis. McGraw-Hill, Inc., New York.

10. Mann, L. D., and L. H. Stolzy. 1972. An improved construction method for platinum microelectrodes. Soil Sci. Soc. Amer. Proc. 36:853-854.

11. Maystrenko, Y. G., and A. I. Denisova. 1972. Methods of forecasting the content of organic and biogenic substances in the water of existing and planned reservoirs. (In English). Soviet Hydrology: Selected Papers, Issue 6, pp. 515-540.

12. Mortimer, C. H. 1941. The exchange of dissolved substances between mud and water in lakes. J. Ecol. 29:208-329.

13. Mortimer, C. H. 1942. The exchange of dissolved substances between mud and water in lakes. J. Ecol. 30:147-201.

14. Nhung, M. F. M., and F. N. Ponnamperuma. 1966. Effects of calcium carbonate, manganese dioxide, ferric hydroxide, and prolonged flooding on chemical and electrocehmical changes and growth of rice in a flooded acid sulfate soil. Soil Sci. 102:29-41.

15. Neel, J. K. 1963. Impact of reservoirs, pp. 575-594. *In* D. G. Frey (ed.), Limnology of North America. University of Wisconsin Press, Madison, Wisconsin.

16. Neel, J. K., H. P. Nicholson, and A. Hirsch. 1963. Main stem reservoir effect on water quality in the Central Missouri River 1952-1957. U.S. Public Health Service, Region VI, Water Supply and Pollution Control, Kansas City, Missouri, March 1963.

17. Patrick, W. H., Jr., and D. S. Mikkelsen. 1974. Plant nutrient behavior in flooded soil, p. 187-215. *In* R. A. Olson, T. J. Army, J. J. Hamway, and V. J. Kilmer (eds.), Fertilizer technology and use. Soil Science Society of America, Madison, Wisconsin.

18. Patrick, W. H., Jr., and A. C. Mahaptra. 1968. Transformation and availability to rice of nitrogen and phosphorus in waterlogged soils. Adv. Agron. 20:323-359.

19. Patrick, W. H., Jr., B. G. Williams, and J. T. Moraghan. 1973. A simple system for controlling redox potential and pH in soil suspensions. Soil Sci. Soc. Amer. Proc. 37:331-332.

20. Pelczar, M. J., R. C. Bard, G. W. Burnett, H. J. Conn, R. D. DeMoss, E. E. Evans, M. W. Jennison, A. P. McKee, A. J. Riker, J. Warren, A. B. Weeks, and F. A. Weiss. 1957. Manual of microbiological methods. McGraw-Hill Book Company, New York.

21. Ponnamperuma, F. N. 1965. Dynamic aspects of flooded soils and the nutrition of the rice plant, p. 295-328. Symposium on the Mineral Nutrition of the Rice Plant. Inter. Rice Res. Inst., Johns Hopkins Press, Baltimore, Maryland.

22. Ponnamperuma, F. N. 1972. The chemistry of submerged soils. Adv. Agron. 24:29-88.

23. Ponnamperuma, F. N., and R. V. Castro. 1964. Redox systems in submerged soils, p. 379-386. Trans. 8th Inter. Congr. Soil Sci. (Bucharest, Romania).

24. Ponnamperuma, F. N., T. A. Loy, and E. M. Tianco. 1969. Redox equilibria in flooded soils: II. The manganese oxide systems. Soil Sci. 108:48-57.

25. Sokatch, J. R. 1969. Bacterial physiology and metabolism. Academic Press, Inc., New York.

26. Soltero, R. A., J. C. Wright, and A. A. Horpestad. 1973. Effects of impoundment on the water quality of the Bighorn River. Water Res. 7:343-354.

27. Takai, J., and T. Kamura. 1966. The mechanism of reduction in waterlogged paddy soil. Folia Microbiol. 11:304-313.

28. Takai, J., T. Koyama, and T. Kamura. 1956. Microbial metabolism and reduction process of paddy soils (Part 1). Soil Sci. Plant Nutr. 2:63-66.

29. Takai, J., T. Koyama, and T. Kamura. 1963. Microbial metabolism in reduction process of paddy soils (Part 3). Soil Sci. Plant Nutr. 9:207-211.

30. Takeda, K., and C. Furusaka. 1970. On the bacteria isolated anaerobically from paddy field soil, Part I. Succession of facultative anaerobes and strict anaerobes. J. Agric. Chem. Soc. Japan 44:343-348.

31. Thimann, K. V. 1963. The life of bacteria, 2nd ed. Macmillan Company, New York.

32. Turner, F. F., and W. H. Patrick, Jr. 1968. Chemical changes in waterlogged soils as a result of oxygen depletion. Trans. 9th Inter. Congr. Soil Sci. (Adelaide, Australia) 4:53-65.

33. Winfrey, M. R., and J. G. Zeikus. 1977. Effect of sulfate on carbon and electron flow during microbial methanogenesis in freshwater sediments. Appl. Environ. Microbiol. 33:275-281.

34. Yamone, I. 1969. Reduction of nitrate and sulfate in submerged soils with special reference to redox potential and water-soluble sugar content of soils. Soil Sci. Plant Nutr. 15:139-148.

35. Yamone, I., and K. Sato. 1968. Initial rapid drop of oxidation-reduction potential in submerged air-dried soil. Soil Sci. Plant Nutr. 14:68-72.

36. Zeikus, J. G., and M. R. Winfrey. 1976. Temperature limitation of methanogenesis in aquatic sediments. Appl. Environ. Microbiol. 31:99-107.

DISCUSSION

PERSISTENCE AND EXTRAPOLATION

TIEDJE: Dr. Kaufman, when a soil chemist begins investigation of a compound in the environment, the first experiment he wants to do is to determine absorption isotherms. Is this an appropriate approach for aquatic sediments?

KAUFMAN: Yes, I think it is a reasonable approach. I think your absorption isotherms have to be established with something which is characteristic of that population.

TIEDJE: Should sediment absorption isotherms be an initial experiment to perform, in a Tier 1 test?

KAUFMAN: I agree with the chemists in that in a Tier 1, I would like to see those six or seven bits of physical/chemical information. Just from the standpoint of having some feeling for the potential for biodegradability.

TIEDJE: You commented on anaerobic metabolism which is an interest of mine. I have a feeling that a lot of people don't know what anaerobic metabolism really is, and how to perform it correctly. I would be concerned that any simple test may not reflect true anaerobic metabolism.

KAUFMAN: I agree.

PLIMMER: One of the likely requirements for soil function tests is that they indicate effects of pesticides on microbial populations. Do you believe we can extrapolate from experiments in the laboratory to the field, particularly with regard to persistence of pesticides?

KAUFMAN: We have to know what goes on with these compounds in the environment. Soil microbes are indeed a part of the environment. From a puristic standpoint, these microbes are just as complex, just as reactive as every other organism in that environment. If you put some chemical out there, regardless of the concentration, it is going to have some effect on some segment of the concentration, it is going to have some effect on some segment of the environment. You can't deny it is going to have an effect; if you do, you deny that biodegradation occurs. It is an extremely complex thing to sit down and measure effects on the total population. But what do you measure? How many *Aspergillus*, how many *Penicillium*, how many bacteria? We all are quite familiar with how complex that gets to be. If we decide as microbiologists that we want to push for that testing, we can create a lot of jobs for ourselves, but is it realistic? I have always supported that processes that go on in the soil are the most important things. If we are going to be asked to do something on the effect of those chemicals on the population, let's look at it from a process standpoint. We know that the nitrogen cycle is important. As long as we don't impair those cycles, I think it is reasonable to say, all right, let's go ahead and use it. If you look at it that you can change the population, even if you lose something out of the nitrogen cycle, is there another part of the population that in the absence of that chemical or another organism, can replace that other organism in the cycle?

PLIMMER: Do you know of any case where a deleterious effect in the laboratory has actually shown up in the field in these microbial function tests?

KAPLAN: I can't answer that question directly, but perhaps I can offer something to help. When we evaluate some of our treated materials, we use soil burial. The soil is maintained in the specific way, it is made up in a specific way, and it is kept under relative humidity conditions in a tropical chamber. These pans have had soil in them now for approximately 30 years, and they have been amended about 20 years ago. These soils have received any number of toxic compounds within impregnated material. You should think that over this period of time as it is reflected in the functionality tests, we should see some differences. But to this day, if you put an untreated fabric into the soil and use tensile strength as your criterion, the untreated fabric loses 50% of its tensile strength between 3 and 5 days. The functionality of that soil, as measured by a physical measurement on a susceptible material, is there. We haven't characterized the population, but it, in fact, supports what you said.

CHOU: Preparation of the tested material has a tremendous influence on the biodegradation rate. When you are dealing with water and insoluble test substances how do you deal with this one problem? Are you using acetone? How do you insure that you have mixed the substrate homogeneously into the soil? Could you comment on this, please?

KAUFMAN: Whenever possible we will put the compound in the water. Yes, we do add pesticides to soils with ethanolic or acetone solutions. We avoid benzene, although benzene in some environments appears to be a fairly biodegradable compound, but frequently inhibits a soil population.

CHOU: Thank you. If this test system is a liquid, and a substance has solubility of only one ppb, if we add a concentrate of 1000 ppm, what will happen? Are you studying the soluble portion or the insoluble portion?

KAUFMAN: Ultimately we're studying all of it. If you are using a ^{14}C-labelled material, a certain portion of it happens to be absorbed or crystallized or whatever it gradually does come into solution, and become a part of the metabolic picture.

PRITCHARD: Do you think, from what you've seen at this workshop, that there is any need to generate biodegradation information in aquatic systems once it has been generated from a soil study?

KAUFMAN: I am not quite sure what you mean. Are you saying that a soil study, such as I do, is adequate to protect an aquatic environment? Or are you saying that the types of things we do in soil will be adequate for the aquatic system? Those are two different things.

PRITCHARD: I agree. Could you answer both of them?

KAUFMAN: I think we are both making it complex. I think there is a certain amount of information to be gained from a soil study which can be applied to the aquatic system. However, based on a lot of what I have heard this week, an aquatic study may generate information which could not be obtainable by extrapolation from a soil study. If you approach it from the standpoint that what you put out in the field gets washed away along with the microbes, then a certain amount of degradation is going to go on while the suspended material is still a recognizable soil particle. Whether we can get that specific in measuring the degradation of a compound in an aquatic environment is a difficult question to answer. When the compound gets down into the sediment, you get into a different type of mineralization. Then, we probably lose some of our ability to project from one environment to another. If you have to make a projection, maybe it should be more on a class or type of biochemical process which could be the same for both environments. If it is degraded under a reductive environment more rapidly than in an oxidative environment, perhaps in sediment it will go much more rapidly. I am really reluctant to go very far with projections from soil to aquatic environment. It's like extrapolating from test tube reactions; you know something happened, you have an idea of how it happened, but from there you've got to put it into the context of environmental conditions.

PRITCHARD: Taking what Art has said in terms of analyzing the environment and its effect on processes, are soil microbiologists much farther along in determining this? Or are our aquatic systems special enough so that we need a whole new set of approaches? It appears to me that a lot of soil studies can be done relatively simply. Putting a compound into a soil sample often generates a result that can be environmentally useful right away. In aquatic environments, it may not be that easy, simply because of immediate dilution.

KAUFMAN: I think we know a lot more about soil than we do about aquatic or marine environments now. I haven't paid much attention to the history of aquatics or marine environments. But I feel that soil microbiology has got a much greater wealth of information at this point. Judging from some of the things I hear you people dealing with here, I think you have got a whole new set of problems.

KAPLAN: I think you first have to establish the correlation. It is bad to assume this is going to happen. I will give you a very specific example. We are concerned with what happens to TNT in the environment in soil, in water, and particularly in our instance, in biological processes as a pollution abatement process. As such, we have studied the fate of TNT aerobically and anaerobically. We have identified the intermediates; we've done all the tagged studies. We've looked for ^{14}C in CO_2, in cellular constituents, in sludge, in condensates, and all of that. We've found in two instances where we have aqueous studies that generally, the same course of action in the soil is found in a biological pollution abatement system. But more recently the question has come up, if you take TNT-contaminated materials and compost them, as you would in an agricultural composting, do you now expect to find the same sequence of events? Again, your first thought was, well, we've studied this thing fairly well, both in the field and in the laboratory. You say, no, it shouldn't be much different. Then you stop and draw back, because there you have the effect of elevated temperatures. You get another set of organisms coming into that environment which is, I understand, the primary degraders of cellulose, the Actinomycetes. So extrapolating from one environment to another, only with extreme caution, and only as a guideline. I do think that you have to establish the correlation, and in fact, you find that you can generally use it advantageously, then fair enough. But again, it's a situation where you tread very softly.

TIEDJE: I'd like to comment on this as one who has done work both in soil and aquatic environments. We always teach microbial ecology from the point of view that microorganisms in nature, and their processes, are very similar; it's only the details that are different. We call an aquatic environment just an excessively dilute soil. There is one basic difference, of relevance here, and that is biomass. It's much lower in an aquatic environment. That's the principal difference. Another point of difference can be the lack of particle surfaces which can often be a moderating effect. The question was raised earlier on the testing for toxic effects on microbes. I would certainly agree that processes are the focus, but I am even wondering if that's worth the resources required, particularly for the soil environment, because there is so little active material in the soil solution, and the microbes have adapted to exist in a rather harsh environment. You really don't have much of a toxic effect. I think the literature bears this out. In terms of development of toxicological testing methods, I think approaches can be somewhat similar for both environments. Soil microbiology has more information because it has been in existence for a longer period of time. However, the aquatic environment is much simpler; whenever we want to try an experiment to examine for a basic principle, we always use an aquatic environment. In soil you never know the microhabitats.

JOHNSON: Are you familiar with the EPA test called the soil/catfish/water test? This is frequently required of industry. I thought this was such a happy marriage right now, you might like to comment on that particular test. The test operates in this particular fashion for a number of pesticides. I think it is required in some situations on other materials. The chemical of interest is incorporated into the soil; it is covered with water; then a catfish is introduced, and one measures persistency. I was wondering if you have heard of it, and would you like to comment on its relevancy? It is a relatively expensive test.

KAUFMAN: I claim ignorance as far as whether the test was a requirement or not. I wasn't aware that it was. By the same token I will say that we did it with one chemical. We put that chemical into the soil, put the water in, put the catfish in just as you have described. We found we had toxicological problems we never really anticipated having. We have not gone back to examine just what it is. We are in the process of doing that now.

JOHNSON: Well, I was just thinking of the validity of the particular organism. How do you interpret this? Is this in reference to a pond or an aquatic environment, or should we consider it an agricultural soil? Or should we consider this some sort of test of metabolism within a catfish?

KAUFMAN: You could characterize it as a runoff situation of the pesticide. You've got sediment settled out, and you've got a catfish feeding on the bottom.

PLIMMER: I'd like to comment on that or a similar test. I think there was a recent publication in the Journal of Agriculture and Food Chemistry dealing with a similar problem. Once I was in the position of the registrant; we were going along with a program to register 2,4-D dimethylamine salt. A problem arose because of a pond experiment. Residues of radioactive material occurred in fish in a small enclosed pond. It took several years to elucidate this problem. There was a residue which appeared to persist. In this particular situation, you had complete breakdown of 2,4-D, probably to bicarbonate, in an enclosed pond system. Then, there was further uptake and incorporation of the residue, probably as bicarbonate or whatever, into a metabolic pool. This was incorporated into tissue and structural material, and it took a long time to establish that the radioactivity was actually present in lipids, amino acids, etc. The question is, what's a residue? I think this becomes a real philosophical question. In fact, you've got residues, but they may be metabolites of metabolites. That kind of test can be awfully complex.

KAUFMAN: Another example: I've done a whole lot of studies with a compound in the soil. I knew what happened to it in the soil. A compatriot was working on his plant uptake in metabolism. He knew what happened to it in the plant. He knew which products might possibly come out of the soil which would be absorbed by the plant, translocated, and to what part of the plant. Having done ^{14}C studies, to characterize or identify all the ^{14}C activity which came up into the seed or calculate it as percent parent material. If they calculated this as percent parent material, they were above the tolerance levels for that compound at that time. But having put all the knowledge from the various aspects together, we encouraged them to take the seed, tear it apart, and examine the amino acids. That's where the ^{14}C was, simply amino acids. But the compound went into the soil, was metabolized, taken up into a product which the plant further converted into a natural amino acid.

SHARP: Dr. Plimmer answered in part some of the queries about the test. I can assure Don Kaufman that the catfish/static test is a requirement. It's called a bioaccumulation test, but what is asked for is a complete metabolism study at every fish sampling period. If you do not have bioaccumulation in the organism, and you are running it at one ppm in the water, it's technologically impossible to define that metabolism. There's a second test, a flow-through test with labelled material. At each harvest, the complete metabolism picture is supposed to be defined. One things both tests do tell us, and that is that Carbon 14 has a very long half-life.

KAUFMAN: This is one of the problems of working with ^{14}C; it has been a great boon to science. In terms of environmental chemistry, it has become quite a handicap as well.

TIEDJE: Dr. Gunnison, I have a question with regard to ammonium production. This might be taken as evidence of dissimilatory reduction of nitrate to ammonium. I noticed that it did not occur when you had the iron and manganese in the system, presumably because the redox was too high for that reduction. Is that a correct interpretation?

GUNNISON: Well, there is probably ammonia accumulation still going on while we were getting our initial manganese reduction. It did plateau after awhile, but we did not look at the particular phase. We did not see how much this nitrogen was being tied up by the "bugs." So that is one thing that I can't extrapolate. When we spiked the system with a nitrate later we did not get the accumulation of ammonia; however, manganese and iron reduction was going on vigorously.

TIEDJE: In your system you had a fairly large amount of organic nitrogen which would be mineralized and thus a source of ammonium. But there is growing evidence of the importance in anaerobic systems of the reduction of nitrate to ammonium. In the February issue of Applied and Environmental Microbiology there are two papers dealing with this subject.

GUNNISON: I am not going to deny it but without a tracer you can't tell.

TIEDJE: Very correct. I would also like to make a comment on measurement of redox. I think people sometimes measure redox much as you would measure nitrate concentration or pH. Because of the chemical basis of the redox measurement, it can only be a ballpark estimate in samples with complex chemical couples. My point is one of caution. Use it as a ballpark estimate rather than a precise value.

GUNNISON: I might also add that even with a more stable system such as an actual sediment rather than slurry like we had, you had to let that probe sit in place for 12 hours before you had a stable reading. We've gotten into a number of arguments with other people from EPA, Georgia, over whether this is valid reading.

POOLE: Did I understand right? You left your redox probe in system for 12 hours? Do you feel certain that you were measuring the redox of that environment or the redox on that probe?

GUNNISON: Do you mean did the probe become fouled?

POOLE: Yes.

GUNNISON: They were very clean when we took them out. We've had problems with that in other systems. We have a larger system, 12 liters; the smaller system does not have the capability of taking all the probes and they do get fouled up. It is very obvious that material has accumulated on them. When we take our probes out, we clean them using a chromex solution to get the bright platinum surface. These Eh probes are our own making, and references are given in the paper. They're fairly clean when they come out of the larger system. I am fairly certain because of the swirling that goes on in the system that they are not being fouled up.

POOLE: We've found with marine sediments that this drift is a great problem. You put the probe in, watch the needle drift down, down, and down. Our standard procedure is to make our measurements after five minutes when the drift stops. But if we don't clean our probes after every measurement, you can guarantee the wrong result. That's with sulfide systems.

GUNNISON: I agree. Sulfide does poison the things rather seriously. We have, on occasion, re-oxidized our system. The thing goes up immediately with no problem. So I would say it's responding normally. Whether some microfouling might have caused some small aberration, I don't know. Our findings are also repeatable when we've run the experiments over again. That's another point in its favor.

BRANNON: To maybe help answer your question, Nigel, we found the electrodes to still be very bright after incubation. I also took them after 40 days of incubation, and checked them in a range of buffered solutions and known Eh and we were still getting a very good response in a fairly reasonable period.

GUNNISON: I have heard a number of complaints as to the dearth of chemists attending this meeting. Mr. Brannon is a geochemist, I think he is qualified to answer that.

TIEDJE: Now, we have an opportunity for open discussion on persistence and extrapolation.

BOURQUIN: Just a question addressed to the panel or anyone else to start some discussion. This week we've talked a lot about methods to look at biodegradation. We talked a good bit about looking for the positive results. One of the questions that was posed in the goals of the workshop was how many or what kinds of tests are necessary to say a compound is persistent? How many negatives do we need to make that decision? The compound is shown to be degradable in certain environments but maybe not in others. What parameters do we use to tell us when this many negative results is really enough to say a compound is persistent?

TIEDJE: Our Task Group discussed some portions of this last night. One of our recommendations for length of test will be two half-lives or one year, whichever is longer. But this doesn't say how many tests are really necessary. The ultimate test would be in a subsample of a habitat of interest.

KAUFMAN: With that bit of coaching, I will answer. I am inclined to agree with that--a couple of half-lives or longer. I think we need to define what longer is. Having assisted in the development of some protocols for OPP, we came up with the philosophy that perhaps two or three half-lives would be adequate, or longer, should be one year whichever came first. In other words, if you go through two or three half-lives in a few months, you are home free, but if you go beyond the year, then you've got other kinds of problems. Rather than wait to get a single half-life, which may take a couple of years, stop at the end of the year and go on to the other problems you anticipate this chemical having in the environment. I would agree with Jim that some fraction of the environment of interest is the best place to test it.

DAUGHTON: I'd like to say something concerning rates which has been talked about a lot and brought down the wrath of EPA. I think there could be a danger in getting hung up on determining the rates whether they're relative or absolute or whatever. They can't supply the information concerning what could possibly happen to a compound in the environment as can our understanding of concepts and processes which can occur. A specific example: you can do a lot of work trying to figure out rates of dissipation, disappearance through metabolism, or whatever, of organophosphate pesticide and its breakdown products. These data have absolutely no relevance at all if one doesn't take into consideration other parameters, especially that of concentration of inorganic phosphates. If inorganic phosphates are present you don't get any metabolism; if it's absent, it possibly gets metabolized. I think this is where the major value of modelling comes in. Not in predicted value, but in the way it constantly brings up new ideas, brings to the attention new things to be taken into consideration in determining how we are going to predict what's going to happen to a certain compound.

DiGERONIMO: I have a question for Jim. If some political bodies are going to regulate some compound and some other organization is going to turn in a fate study or persistence study, could you speculate on whose data you would take if based on concentration with regard to persistence? Also, how low do you think you should go in terms of concentration?

TIEDJE: Could you repeat your description of the two sources and the information?

DiGERONIMO: Well, somebody, say EPA, may turn in work at a concentration of one ppm or 40 ppb and you see persistence that lasts for 20 days. But then somebody else, the chemical company, turns in other findings that say our concentration at 40 pptr lasts one day. Okay, is that considered a persistent compound then, or what?

TIEDJE: From our Task Group discussion of persistence and extrapolation, we feel that at least three concentrations need to be examined. These should bracket the extreme that could be expected to occur in the environment of interest. Hopefully, that would then cover the case you're dealing with.

DiGERONIMO: The last part is whose environment would you validate this on? If I am Dade Chemical Company, the EPA is doing it one way and I'm doing it another, and we get conflicting results, how do you extrapolate that?

PLIMMER: I'm sorry that I came into the meeting rather late to catch up with the gist of this. But the question I ask is, when does it become necessary to determine persistence? That is, if we look at all the compounds coming under the Toxic Substances Control Act, surely we've got to make a selection before we start discussing the question of persistence. In pesticides, where we have a lot of experience, it's quite clear that we have to ask that question. Now, I think, somehow that there's got to be a hierarchy of questions, a tier system or whatever. Persistence may be one of those further down the line. This may have been discussed before, but the range of organic substances that we're going to look at will presumably include polymers and things like fiberglass boats or whatever. I would hope that some of them would persist for a long time. If my boat degrades in the environment, Paul, I hope you'll be in it with me.

DiGERONIMO: I am not sure if he answered my question.

TIEDJE: No, we didn't answer the question. I guess it's very difficult, but if I were making regulatory decisions, I would have to evaluate how near the test environment was to original habitat. This brings in the question of diversity of the habitat, and we felt in our discussion group that because of the great diversity of habitats, one could not extrapolate rates. One could get an impression of what is expected based upon the rank of the test chemical in the degradation test compared to the behavior of it to some known compounds.

DiGERONIMO: But the main problem is if you have two conflicting reports, one like from a government agency whose job it is to protect the environment, and another from a company whose job it is to make a profit. How are you going to handle that decision? For example, last night we had two conflicting reports on Kepone. I was just wondering, how would you handle something like that?

KAPLAN: Well, I think you're premature in some of these suppositions. It may be that both sets of data are equally valid from the company and the regulatory agency. Of course, you look at the merits of the test to see whether they are valid. Furthermore, you can't extrapolate unless you have a field base for recovery or a past experience. If we say you can't extrapolate because rates as such have no meaning in the environment, because of all the perturbations, then you can't set a level unless you have some experience. With old chemicals, fortunately, we are able to do this to a degree. With new chemicals, you simply are not going to be able to do this until that accumulates in the environment. But with regard to persistence, if the compounds are going to be toxic, for example, then you don't want it. Your decision would be you're not going to have it in the environment. Of course, then you begin to be concerned whether it's an all or nothing, tolerance levels. I think at this point for new chemicals, or for chemicals with no experience, you're setting up hypothetical situations that I think you can't address until you're faced with an exact situation.

KAUFMAN: I'd like to make a comment in defense of industry. That is, yes, industry is interested in making a profit, but they're also interested in protecting that profit. And they are not about to let that chemical go out there, at least in most reasonable situations, without having some basic knowledge. For the most part, we recognize, they're doing a darn good job. Yes, there are those who are not, and I think as time goes along those are becoming obvious. It's human nature, some people do a good job, and there are others who don't. By virtue of many characteristics, you understand and recognize where some of that is. Something else which we talked about again in formulating protocols for OPP is the use of a benchmark system. The benefit of the benchmark system, in this case, is that along with the chemical that you're really concerned about, you have another standard that has already been studied, and we know a lot about. In the system that you're looking at, you watch the behavior of that chemical just as closely as you watch the one that you're primarily interested in. This has several advantages. It gives you a measure of where your chemical is, and how it's relating to the standard. It also gives the person judging your work a unit of measure as to how well you did your work, or what your environment was like, etc.

BETTINGER: It seems to me that pesticides known to persist in the environment have garnered most of the attention here this week, at the expense of all those other compounds that are less refractory. The efforts of the task groups seem to have been channeled into following the fate of pesticides into every ecological niche possible, and not on the general question of persistence of all compounds. It's a mistake to approach a definition of persistence and the means to test for it using pesticides as the only model. We have to be open to assessing all compounds in a rational manner, particularly when it comes to screening methods for persistence.

BAUGHMAN: I want to make a point that somebody has to make at this meeting, it may as well be me. That is, I'm just a little bit dismayed at the aversion to extrapolation that appears to be among this group of people. The truth is, we're going to extrapolate. EPA is going to extrapolate, industry is going to extrapolate, every scientist in this room extrapolates all the time. It's a moot point whether or not we're going to extrapolate; the answer, I believe, is unequivocally yes. The question is, what are the grounds on which we're going to base the extrapolation? One of the things that I find rather difficult to cope with is that we're extremely concerned about how to develop tests. Yet, we haven't decided on the rationale by which we'll analyze the data and how we will extrapolate it. Every test, whether it be in the basic sciences or whether it's for some environmental purpose, invariably is used for extrapolation. In most cases, if data do not have some extrapolational values, they are not very good. This is even true in the laboratory; if we make the calibration curve for a chromatograph, we use it to extrapolate between the points that we put on the curve. In view of the difficulties of defining terms like persistence and questions about how we are going to develop tests, it seems very clear that we have to give a lot more attention to the rationale we're going to base our extrapolations on, and quit worrying about whether we will extrapolate.

TIEDJE: Good point, I would have to agree. I think by the very nature of scientists, we tend to be conservative in interpretations of our results. It is very difficult to face the hard fact of having to make a final decision on a set of guidelines. You know we're used to 95% confidence intervals; it's difficult to merge the two positions.

BAUGHMAN: I agree with you. I think a significant factor here is not whether the decisions we need to make for these purposes are scientific, but rather how can we make the best decision with a reasonable amount of data? It seems to me the kind of data and decisions that we need, as scientists, is different from what we need in order to make a good value judgment about whether or not a compound is to be used in the environment. We simply can't afford the kind of data to make scientifically defensible decisions on every compound.

DAUGHTON: Just a comment on the standard curve analogy. I don't think that many people like to extrapolate to community interpolations. I don't know if that would be of much use to us. Hap, one thing I wanted to say concerning rates. It's possible to figure out a rate for the worst possible world situation, whatever that is. Then maybe it is possible to use that as a basis for determining what an application or effluent rate should be. So if you end up determining rates for the best of all possible worlds, then we get into a lot of trouble.

PRITCHARD: I agree wholeheartedly. On the one hand, I think the more input we have into the determination of a rate in terms of environmental effects, the better the rate measurement will be. On the other hand, we can't go on and on forever doing these effects' measurements; somewhere we will have to draw a line. As George indicates, we're at the point now where we haven't got a hell of a lot of time. We will have to take what we can get. We'll have to put as much effort into looking at all variables to make sure a rate measurement is valid, but at the same time we must go ahead and use what we've got now.

GUNNISON: I'd like to point out that there are occasions when you don't have the data. You may never have it. Take the case of a proposal to build a reservoir in some long forgotten mountain valley, and you're called upon to write an impact statement for water quality. You may be so fortunate as to have several reservoirs or lakes in the neighborhood which you can run out and gather data on. Then extrapolate, comparing the inflowing stream quality and the quality of the floor that's going to be flooded when the reservoir is filled. You may be so unfortunate as to have no example. In that case, then that math model may be the only way to go about it. I'm not waving the flag for math methods but here's a case of extreme extrapolation.

BAUGHMAN: I think there is a different kind of data than what we have talked about that is extremely important to those of us who would like to extrapolate occasionally. And that is, there is a tremendous need for environmental studies that give an idea of the range of variability that one finds in the environment. For example, it's one thing to have to make an extrapolation with respect to some parameter that varies a factor of two in the environment, but quite a different matter if it can vary over seven or eight orders of magnitude. And we really have very little data about the different kinds of environmental parameters and the ranges they cover. For example, I believe Dr. Slater suggested that the K_s values for microorganisms are subject to change. But we really know little about the magnitude of rate constants for microorganisms in water or the kinds of ranges over which those parameters can vary. It is extremely important, if you can't measure the parameter, to be able to put a range on it. Only in a very few cases at present can we do even that.

TIEDJE: I would like to introduce one other topic that I think needs some consideration at this meeting. That is the future direction, or future optimization of biodegradation research. What I mean is, what should be the interrelationship between government, industry, and university groups? This is in terms of topics of research, resources for research, and publishing information from research. I'm concerned that we get the best conceptual advances in biodegradation research. My impression of the field as a whole is that this has not occurred. In my own case, I have considered leaving biodegradation research, because it's become primarily a government and industry activity. On the other hand, I am interested in seeing that the conceptual advances occur and that there is some check on those two organizations going their own way.

SUMMARY

Chairperson, Stanley Dagley

TASK GROUP REPORTS

TASK GROUP I: TRANSFORMATIONS IN AQUATIC ENVIRONMENTS

C. D. Litchfield, Chairperson

The purpose of this task group was to determine whether: biodegradability would be the same in soils as in aquatic environments for natural organics; would the rates of biodegradation be equivalent; and what distinctive factors in the various sedimentary environments might affect these rates. A further question arose concerning the concentration or threshold level of substrates and their effects on microbes.

In regard to general biodegradability in terrestrial versus aquatic systems, there seemed to be a consensus on the following two points: If you do not find decomposition in an activated sludge or soil system, then *probably* you would not find degradation in aquatic or marine environments; however, just because decomposition is occurring in these model systems, it does not mean that degradation will occur in aquatic environments. This leaves us with the question of rates. There are likely to be major rate differences between soil and sediment because they differ primarily in: the component microbial flora, with marine sediments containing fewer fungi, *Streptomycetes*, *Bacillus*, etc.; a greater potential for anoxia in sediments, i.e., lack of oxygen, high H_2S, high NH_4 levels; less susceptibility to temperature extremes in marine sediments; and water normally is not a limiting factor in marine sediments. Also, in general, soils and aquatic sediments differ in that water is not a limiting factor in the surficial layers in the latter environment. Hence, water and compounds move both into and out of sediments, while in soils, movement is generally downward. Thus, microbial degradation in sediments can drastically affect concentrations in the water column by altering the purely abiogenic equilibrium. However, in both systems, the prevention or activation of microbial degradation and decomposition rates will be affected by sorption and interface phenomena. Sediments which can become anoxic will most likely have decreased decomposition rates. The overall end result may not be different as it is likely that alternate pathways have been evolved by anaerobes.

Because of the distinctive property of aquatic environments, the dilution by an aqueous environment, it is likely that rates will be slower than in terrestrial or sludge systems. Distinctive characteristics of aquatic environments can only be considered if we separate freshwater and marine water. In freshwater systems, specifically lakes, development of seasonally induced anoxic hypolimnion and severe temperature fluctuations produce major shifts in microbial types, biomass, and activities. In both systems, nutrients (nitrogen, phosphate, oxygen) can be limiting. However, in marine waters, the organic and nutrient loads are routinely very low because of the sheer dilution

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effect of the volume of seawater. This nutrient limitation can have the direct effect of preventing microbial decomposition. It is also possible that the decomposition of organic compounds will be accelerated simply because there is a deficit of organic carbon.

Regardless of the aquatic environment, several other parameters must be considered, including current patterns, hydrography, temperature, salinity, and season. The first two, currents and hydrography, affect nutrient distribution and pollutant dilution. Bacterial numbers, generic types, and activity levels are known to vary with season, temperature, and salinity. Salinity and temperature are likely to be more important in estuarine than in coastal zone situations. In estuaries and coastal zones, tidal influences will affect bacterial numbers and the physical-chemical characteristics of beaches and mud flats.

A final consideration of the task group was to assess the effects of concentration and threshold. A conceptual framework was developed in which the threshold concentrations of a compound are the minimum and maximum concentrations of the compound above and below which microbial degradation can no longer be detected even though other necessary factors (pH, Eh, oxygen, nutrients, and biomass) are favorable.

In concluding this philosophical analysis of environments, it was observed that something which is not degraded will most likely end up in sediments, on beaches, etc. In considering the rate of biodegradation, the environment into which the compound will most likely be introduced should be mimicked as closely as possible because the unique properties of that environment will affect the amount of biodegradability noted. It is possible that any compound could be degraded but this is not likely at rates that will prevent accumulation. After all, if marine sediment microbes rapidly degraded all hydrocarbons, we would have no problems evaluating offshore drilling.

Task Group Members: T. Blackburn, M. DiGeronimo, G. Floodgate, M. Hood, A. Ivanovici, J. Jeansonne, H. Kadota, D. Laskowski, C. Warnes, R. Wright

TASK GROUP II: BIOCHEMISTRY OF MICROBIAL DEGRADATION

D. T. Gibson, Chairperson

The reasons why biochemists study the biochemical aspects of microbial degradation are diverse and, in many instances, only tangentially related to environmental pollution. As a general statement it may be said that the goal of scientists working with pure cultures and isolated cellular components is to understand various cellular processes in terms of our current knowledge of chemistry. Microorganisms, particularly bacteria, have proved to be particularly suitable for such studies and the current knowledge of the pathways of intermediary metabolism, and the enzymology and genetic regulation of such pathways is extensive. In contrast, the intimate details of the degradation of molecules whose structure is far removed from the compounds of intermediary metabolism is less well understood. For the purposes of this workshop we feel that we should point out that in most instances detailed biochemical studies are not directed toward providing a full explanation of environmental interactions. But, until techniques are available to conduct such studies under environmental conditions they could be viewed as an important component of the current attempts to explain the fate and effects of certain classes of compounds in various environmental habitats.

In very general terms it may be stated that the cycling of carbon in nature predicts the existence of organisms that can degrade molecules that are produced by biosynthesis. This is because the organisms have had sufficient time to evolve the requisite enzyme systems. In contrast, there is no valid reason to expect that microorganisms are able immediately to metabolize compounds that bear little or no structural resemblance to naturally occurring compounds. Thus, for many of the synthetic chemicals produced by modern technology it seems probable that they undergo biodegradation since they fortuitously contain structural features that are common to naturally occurring compounds (for example, the chlorophenoxyacetic acid herbicides which can be related to those chlorophenols found to occur naturally).

The task groups spent many hours discussing how pure culture and mechanism studies could be useful to present and future investigations at the environmental level. One of the conclusions reached was that there are hundreds of easy questions and very few definitive answers.

1. What is presently known about the degradation of compounds in relation to chemical structure?

At the present time the amount of information is limited, although certain classes of compounds have received intensive investigation. Nevertheless the group felt that the available data could provide a fragmentary but valuable basis for assessing the potential susceptibility of new chemicals to undergo microbial degradation. Such assessments have obvious implications for regulatory decision-making and the design of new synthetic compounds. While purely a priori chemical considerations will probably never replace direct studies on the environmental fate of novel compounds in assessing environmental impact, such considerations have both heuristic and intrinsic value for attempts to answer such questions such as: To what extent is a given compound likely to prove recalcitrant? What classes of metabolites may be formed? Is the transformation of the compound likely to be conditional upon environmental factors, for example, the availability of oxygen?

We should point out, however, that even if such an approach should prove to be useful it is doubtful whether it will ever be possible to obtain completely reliable and consistent predictions on the decomposition of many organic compounds. This is due to our inability to assess adequately all environmental parameters which can place constraints upon the biodegradation processes outside the laboratory.

2. What is the value and relevance of pure culture-mechanism studies to environmental concerns?

We perceive a number of ways in which such information can support and inform about biodegradation in the environment:

a) On purely formal grounds one can recognize from the structural features of organic compounds the possible chemical reactions which they may undergo. A knowledge of biochemical reactions used by different microorganisms can then reveal the alternative strategies available for biodegradation. When these are subject to close scrutiny by characterization of the purified enzymes which catalyze individual reactions, properties such as specificity, cosubstrate, and coenzyme requirements can reveal whether certain environmental factors can limit their action. For example, the role of oxygen as a cosubstrate for so many enzymes of the monooxygenase and the dioxygenase classes is now recognized in the use of mechanical agitators for aeration of lagoons used in waste water treatment.

Factors such as permeation and mechanisms of enzyme derepression are important considerations in microbial metabolism, yet we know the ranges of specificity of these systems in only a handful of situations. Explanations of the failure of cells to modify or degrade certain compounds may be found, therefore, not only in terms of the availability and specificity of appropriate enzymes, but also with respect to permeation and enzyme induction mechanisms.

It should be stressed that this type of information is all qualitative in character, that is, it will not provide an estimate of the rates of breakdown of compounds. Its potential is in revealing the reactions that may occur, yet does not guarantee that they will. Suggestions as to the organization and systematization of a body of information about degradation mechanisms possessed by microorganisms will be found in a later section of this report.

b) Relevance to Environmental Fate Studies. A body of information describing the range and variety of microbial conversions which may occur has great value in guiding studies of the environmental fate of organic compounds. For example, the identification of degradation products is facilitated by a knowledge of possible reactions. Such conversions as polymerization, oxidative coupling and conjugation constitute a range of alternative reactions which must also be considered along with degradative reactions. Detection methods for such compounds would then be developed as needed.

c) Relevance to Mixed Substrate-Mixed Culture Systems. The molecular events which underlie the various kinds of mixed substrate transformations collectively referred to under the heading "cometabolism" undoubtedly have their explanations in terms of substrate and inducer specificities. If these processes are to be of value in facilitating degradation it follows that a better understanding can lead to more effective use. The interactions between different components of stable communities of microorganisms (consortia) have become a focus of interest in recent years. Explanations of the specific chemical interdependencies in communities effecting degradation will derive in part from studies of the individual microbial participants in pure culture.

d) Relevance to Testing Methodologies. Knowledge of the biochemistry of microbial degradation can provide rationales for design of appropriate testing procedures for assessing biodegradation in the environment. At the same time, it can reveal limitations of existing methodologies. For example, measurement of amounts of $^{14}CO_2$ released from a ^{14}C-labelled organic compound must be assessed in relation to the pattern of label present in the parent compound, the metabolic fate of individual carbon atoms and the extent to which assimilation may occur. In measurements of oxygen consumption to show biodegradation, care must be taken to recognize that uncoupling of oxygenation reactions without substrate attack may be responsible for the observed uptake.

e) Relevance in Guiding Studies of "Directed Evolution." While the modern techniques of molecular biology have great relevance to research in areas of biodegradation, it remains problematical whether the construction of bacterial strains carrying multiple degradative plasmids contributes a greater degradative capacity to the natural environment than is already present. Alternative approaches to development of improved "degrading" strains can be envisaged which depend upon alteration of enzyme specificities and upon regulatory changes which increase the amounts of enzymes manufactured.

3. Limitations

a) Past degradation research has concentrated on pure culture studies using, for the most part, bacteria; some fungal work has been conducted but essentially no studies of other groups of unicellular eucaryotic organisms have been made. Failure to examine such groups in detail is seen as a shortcoming of pure culture-mechanism studies.

b) There is no information on the existence of microbial flora specifically equipped to utilize and thereby degrade organic substrates at low concentrations. This is seen as a limitation of the present work.

c) There is a deficiency in our present knowledge with respect to the biodegradative abilities of specific groups of microorganisms other than those which are usually isolated by conventional enrichment procedures. Thus, organisms which are found in uniquely characteristic habitats of the aquatic environment generally have been overlooked. To cite one example, the catabolic activities of marine microorganisms have been little studied yet they are found in an environment known to contain the greatest amount and variety of organohalides of natural occurrence. Can we afford to overlook the possibility that such organisms may be more adept at breaking down synthetic organohalides? A second example relates to the actions of organisms occupying strictly anaerobic habitats. Whether they derive energy by fermentation or by anaerobic respiration our knowledge of the biochemical processes involved is grossly deficient.

4. Polymers

Structurally useful polymers, both natural and synthetic, present special problems to microorganisms for their biodegradation. These include chemical structure, insolubility, compact three-dimensional array and size. Studies on the biodegradation of lignins and ligno-celluloses have not yet revealed the mechanisms for the depolymerization and mineralization of these molecules. Lignins, like many synthetic polymers, do not possess readily hydrolyzable bonds, and serve plants by their structural properties and durability. A considerable period of time can elapse before the carbon of lignins becomes susceptible to a variety of environmental weathering and biodegradative processes for its return to a dynamic portion of the carbon cycle. Some synthetic polymers have chemical structures that bear no resemblance to naturally occurring molecules, and little, if any, evidence is available to show any biological decomposition.

In the context of this section, mention should be made of the fact that we would benefit from the inclusion in studies of catabolic pathways, of experiments to test effects such as changing pH, temperature, salinity, etc., on overall degradative capacity.

5. Systematization of Information

The vast literature of biochemical transformations is scattered in a variety of basic, applied, technical and environmental journals, not to mention in-house reports of manufacturing industries and governmental agencies. There seems to be no reason why this cumulative knowledge could not be systematically arranged and classified for general use and easy access. This could be done in terms of the chemical structures of compounds, such as functional groupings and their arrangement in each molecular structure, and what is known about their biological (and physico-chemical) transformations observed in the laboratory and field. Such a compilation would be facilitated by the use of computers and the application of Wisswesser procedure to denote the structures of compounds by unambiguous line notations, as is already done for those interested in structure-activity relationships, and which are currently in use in searches for new drugs. A compilation of this kind could readily be designed to predict what specific organisms and environmental conditions might allow biotransformation of a compound under study, as well as provide the relevant literature references.

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TASK GROUP III: COMPARTMENTALIZATION IN AQUATIC ENVIRONMENTS

G. Chesters, Chairperson

The use of the term "compartments" led to semantical problems and the term was largely discarded during the course of the task group deliberations. Discussion centered on the following portions of the aquatic environment: 1) surface microlayers, 2) areas of anoxia, and 3) bottom and suspended sediments involving pollutant adsorption. Consensus was achieved that any evaluation of the hazards of toxic organic chemicals must take into account the effect of these conditions or processes on rates and types of degradation.

Continued research directed toward evaluation of the role of surface films on microbial and chemical degradation and on the residence time of the compound in the surface layers should be assessed. If, in fact, organisms and pollutants are concentrated in surface microlayers, it is anticipated that this set of circumstances should markedly affect the rate of degradation of pollutants. No clear evidence of generally enhanced degradation of pollutants in this zone has been offered, and at this time it is difficult to assess the importance of surface microlayers in ridding the aquatic environment of toxic pollutants. If residence times of pollutants in the surface microlayers are shown to be short, the effect on degradation processes is likely to be limited and the phenomenon could probably be ignored. Conversely, if residence times are long, then added significance should be given to the role of surface microlayers in purging the aquatic environment of hazardous chemicals.

Clearly, zones of anoxia occur either in the water column itself or in the sediments of aquatic environments. Research needs to assess the significance of anoxia in marine and other aquatic environments include: a) determination of the possible chemical/physical changes which might occur to an organic pollutant in a reduced environment, for example, differences in the pathways of degradation of DDT in aerobic and anaerobic environments; b) evaluation of the synergism which exists during the degradation process between physiologically distinct microbial populations indicating that pure culture experiments have little practical value for assessing the biodegradability of pollutants; c) assessment of the possibility that a compound may indirectly cause anoxia to develop or be maintained, for example, by altering the behavior of certain members of the benthic fauna which may result in changes in the rate or type of degradation of the compound and producing metabolic components which could alter the normal toxicity spectrum of that compound, e.g., production of hydrogen sulfide; d) development of rigorous, inexpensive methodologies for providing anoxic conditions which can be replicated and used routinely to study anaerobic degradation.

Adsorption reactions at the surface of particulate matter are likely to affect the rate of removal of toxicants from the environment. In general, adsorption by sediments is governed by the surface area exposed by the particles. However, it should be noted that dispersion of sediments or soils by drastic means, i.e., removal of organic matter by treatment with hydrogen peroxide followed by shaking with sodium hexametaphosphate or by treatment with ultrasound, is not likely to reflect the extent of exposed surfaces in soils or sediments. Other methodologies which simulate the conditions which particles are exposed to in the environment must be developed. Degradation rates for adsorbed pollutants by purely chemical mechanisms are frequently increased over rates for the same pollutant in the nonadsorbed state, i.e., adsorption-catalyzed chemical degradation. Conversely, the rates of degradation mediated by biological processes often are decreased, perhaps due to decreased accessibility of the pollutant to the organism. Photochemical degradation for some compounds is frequently enhanced in aquatic systems containing suspended sediments. Furthermore, rates of volatilization, extent of uptake by plants and animals and transport of pollutants either through soils, overland or down waterways is markedly reduced by sorptive reactions. From a research perspective isolation (methods of sampling) or particulate materials in the dispersed or aggregated form in which they actually occur in the environment with their associated pollutants intact need to be devised. The accomplishment of this task would greatly assist in developing management strategies for controlling the transport of land-generated pollutants to water bodies. Also, methodologies are needed for assessing the types and amounts of bound residues found in soil and sediment systems.

In terms of developing a testing protocol for evaluation of risk incurred in utilizing particular organic chemicals it is clearly not feasible to evaluate the effects of surface microlayers, anoxia, or particle-pollutant interactions on all chemicals subject to review. Some feeling was expressed that an interpretation of the impact of anoxic conditions and pollutant adsorption by sediment is needed even if direct experimentation cannot be incorporated into the testing protocol. For example, based on the some hazard scale a particular compound may be placed in some borderline category, but a decision might be made to give the compound a "safe" designation based on the knowledge that the compound is strongly and almost irreversibly absorbed by soil and sediment particles and this reaction markedly inactivates the compound. From a microbiological viewpoint it was deemed important for the testing methodology to include an evaluation of the impact of a compound under review on such major processes as cellulose decomposition, the nitrogen cycle and perhaps others.

Transferability of information from freshwater to marine environments is urgently needed and the extrapolation of detailed information gathered in small watersheds or water bodies to large regions or major water courses must be undertaken even though the interpretation leads to a marked decrease in precision.

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TASK GROUP IV: MICROCOSMS

F. Matsumura, Chairperson

Microcosms are defined as any experimental setups and approaches to simulate or study the events that actually take place in the environment. Much has been said about microcosms and several symposia have been held to discuss the pros and cons of microcosm approaches in the past, the most notable one being the workshop held in Otter Creek, Oregon, in June 1977. Frankly, this task group has recognized past failure of previous workshops and began the session by finding out the problems first.

The problems in utilizing microcosms approaches have been that 1) there are many different designs, each suiting some purpose; 2) the field encompasses scientists from many, often unrelated, fields of science, such as system scientists vs chemists; 3) the lack of the realization that no microcosms can ever produce the same overall effects as the environment; 4) the lack of reproducible experimental design; and 5) disproportional by high biomass in microcosms as opposed to the natural environments. Above all, item (3) shown above appears to be the most serious problem. In the past, certainly too much was expected of microcosms. Some people expect absolutely quantitative microcosms data identical to that obtained from nature. Other people tried to put everything together in the hope of simulating *all* the reactions that go on in the environment only to find out that such complex systems are too difficult to reproduce, too cumbersome to control each environmental factor, too costly and most seriously of all, yield data which are very difficult to interpret.

On the other hand, one must look at the problem with a positive attitude. After all, there is no panacea in any field, and yet progress has been made in many fields by agreeing on a uniform initial approach. Toxicologists, for instance, have been relying heavily upon the use of rats as the primary screening means, although rats do not give all pertinent toxicity data on human and other important biological systems. Molecular biologists use *E. coli* for their studies on basic cellular function despite the fact that it is not even a eucaryotic organism. These approaches have served as the primary study protocol for scientists in each field, and other secondary and tertiary study materials such as primates and human cell and organ culture for the former and Drosophila for the latter, etc., are used to supplement the deficiencies caused by the choice of the primary studies.

At the same time, if this task group is to make positive contributions, we have to clearly understand microcosm studies, the philosophy behind them, the purpose, the strong points and the weakness of microcosmology, and devise a plan of approach.

Philosophy

Any microcosm system is designed to study merely a small facet of environmental events. Often only one aspect of environmental events can be studied or simulated in one set of experimental designs. While it is possible to design the experiment to ask questions on specific issues (e.g., photodecomposition, etc.), it must be clearly understood that no microcosms would give the data quantitatively equal to the events taking place in nature. All values generated by model ecosystems-microcosms are therefore only valid in a relative sense.

Purpose

Microcosm studies may be used either for screening or prediction purposes so long as their limitations as outlined above are understood. However, microcosms do not eliminate the necessity of field testing or environmental studies. While microcosms studies may be a prominent tool for screening many premarket chamicals, they must not be used for a regulatory purpose, at least at its current state-of-the-art. It is possible that a microcosm approach would give manufacturers the sense of *relative* stability and bioaccumulation potentials for a compound, and thereby aid their decision-making processes to further pursue its course or not. However, microcosmology does not guarantee the actual safety or unforeseen events related to the fate of the compound in nature.

Microcosms: Definition and Explanation

Many people, including scientists themselves, have had trouble defining microcosms. Microcosms mean one thing to a section of science and another thing to others. There are basically four types of microcosms approaches:

a) "Benchmark" approaches where physicochemical parameters such as volatility, water-solubility, partition coefficients (e.g., water-octanol partitioning), etc., are determined and are utilized to predict the probability of environmental behavior of compounds.

b) Single reaction approaches whereby only one set of interactions between the compound and one of the environmental forces, either biological or physical, is studied. The use of microbial axenic cultures, photo-illumination to simulate sunlight and fish accumulation studies in the absence of food organisms, etc., are included in this category. The advantages of such systems are 1) reproducibility, 2) ease by which various environmental factors can be controlled and varied, and 3) usual indication of the presence of the cause-effect relationship.

c) Multicomponent systems, where more than one factor is placed in one system. They range from a small test tube containing nonsterile water to complex self-sustaining model ecosystems which may house various organisms at different trophic levels in constituting an ecosystem-food web. Often only multicomponent systems are referred to as microcosms. The advantages of this type of microcosm are 1) one step closer to the environment, 2) often the data on the ecological interactions can be generated, and 3) multistep reactions can be studied, etc. On the other hand, the disadvantages of multicomponent systems have already been described in this report.

d) System analysis approach. This route of approach to the environmental studies has been made by mathematically oriented scientists such as systems analysts, biostatisticians, etc. In many instances, these scientists use the data generated by model ecosystems or small field studies or laboratory experimentation to predict or determine the rate by which compounds are metabolized and/or transported through the biosphere.

RECOMMENDATION

1) Relative Nature of Microcosm Data. It is most imperative to understand the fact that microcosms approach can only give values which are relative to other materials tested under the same system. That is, microcosms may give the data that one compound, i.e., DDT, degrades faster than another similar compound, i.e., methoxychlor, in the same experimental setup. The system by itself does not necessarily give the value as to how many months or years the compound lasts in one system and this value does not mean anything to others.

To this end the task force recommends that any microcosms studies include a number of reference compounds which serve as the standard for the system. Such standard compounds are selected on the basis of the past knowledge of their environmental behavior (such as DDT, parathion, LAS, etc.) or the similarity of chemical structure particularly regarding their functional groups.

2) Reproducibility. To insure the validity of microcosms experiments, it is imperative that the given system produces reproducible data within a set of experiments. Otherwise, comparison among compounds as proposed above would become meaningless. It must be pointed out here that such a requirement tends to favor small and simple systems over more complex ones. The latter systems certainly have a place in other areas than in the comparative studies as outlined above. Proper controls, such as a sterilized system for microbial studies, are also to be used so as to ensure that no artifacts are involved.

3) Consideration of Biomass. Any model ecosystem which contains disproportional amounts of biomass could give unrealistic data. For instance, if a large animal is placed in a small model system, the animal could consume most of the test compound in a relatively short time span, and thereby minimize the differences among test compounds. In this context, the task force feels that microbial studies are most suited for model ecosystems because of their small sizes in relation to that of the model system.

4) "Benchmark" approaches (including literature survey) are recommended as a prerequisite for model ecosystem studies. There is no question about the phenomenon that physical properties affect the fate of chemicals in the environment. The choice of type and design of model ecosystems could be guided by the physicochemical parameters obtained by benchmark approaches. For instance, if the test compound is known to have a high volatility, studies on air-mediated transport and photochemical degradation would be the logical choice. If the compound is shown to be lipophilic, tests on its bioaccumulation potential are necessary. Data generated during phases of testing the compounds can be utilized in the formulation and calibration of mathematical models describing the fate and effects of the compounds in complex systems. The interaction between experimental and modeling activities can provide for the organization of diverse data and the identification of data needs.

5) Adoption of "Tier" Systems. Since each microcosm approach can only look at limited aspects of environmental events, it is more desirable from the viewpoint of screening to test several simplified microcosms approaches than to rely upon one complex and elaborate model in the hope of getting many answers all at once. These can be open or closed systems and must include physicochemical controls.

Typically a screening process could begin with benchmark approaches (Tier I). The next step is to use simple model ecosystems such as degradation studies by using soil plugs, lake water-containing sediments, studies on bioaccumulation using fish (Tier II); then followed by simple test systems such as degradation studies in the pure cultures of microorganisms appropriate to the recipient system (Tier III). This is the stage where metabolism must be studied. In due course, radiolabelled compounds must be used in the more complete metabolic and distribution studies in model systems. Efforts must be made to account for all radioactivities including CO_2 and other volatilized portions. At the last step, an appropriate complex model ecosystem may be tested (Tier IV). This step is not meant to obtain relative indices among various test compounds, but to study ecological significance.

6) Field or Semi-Field Testing. In the final analysis, field and semi-field (including enclosed outdoor plot and pot tests) testing is an important step in ascertaining the environmental safety of the chemical (Tier V). The compounds which have been advanced to this stage must show great economic promise. In the case of metabolic studies, this is the step whereby the presence of the metabolites identified by the model systems is confirmed and their quantities and environmental significance are determined. Indeed, many environmental conversion products have been identified in this manner. At the same time, it must be pointed out that in several instances some surprises were also found in that certain reactions which looked prominent in microcosms studies have not been found in the actual field test. Thus, the value of small scale field testings cannot be over-emphasized. At the same time, this is the stage where the benefits of data organization from mathematical model development are realized and system analyses can work most efficiently and effectively.

In preparing this report, we had to make an arbitrary decision that we discuss microcosms approaches from the viewpoint of their usefulness in screening processes. Certainly there are many other uses for microcosms studies. Many scientists have been using microcosms as simplified approaches to analyze each factor of environmental forces. Indeed, in many ways, microcosms are nothing but "test tube" or laboratory approaches to field problems and are well accepted methods of studying in science. Microcosms are also useful as predictive and confirmatory, but these aspects are not considered here in depth. However, we have not examined these different uses of microcosms here. "Microcosmology" is a very young field and there are many unsettling aspects. By its very mission to study complex forces of the environment, scientists from many different disciplines have been participating in designing various microcosms. Because of the differences in their training and their mission, many insist superiority of their designs over others. Certainly many are so for their specific purpose. Nevertheless, the important thing here is to limit our scope and define the mission of the committee in order to reach some form of agreement or "consensus." The route we took was to look only at the usefulness of microcosms approaches from the viewpoint of screening "tools."

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TASK GROUP V: METHODOLOGICAL CRITERIA FOR BIODEGRADATION

R. L. Raymond, Chairperson

Introduction

Under the Toxic Substances Control Act it will become necessary to assess the fate and effects in the environment of a large number of chemical compounds. To assist the regulatory agencies in making risk assessments based on protocols of testing for biodegradability, our task force in this workshop has attempted to determine the state of the act with respect to methodology for such testing. It was recognized from the outset that the methodologies to be considered would have to be in the realm of economics feasibility. The goal of the task force was further narrowed to encompass only those methodologies which would have application in the aquatic environment.

General Definition of Methodology

Tier 0 or Tier 1 testing should have a stated objective or objectives which in most if not all cases should indicate whether a pollutant is being biotransformed. Traditionally such measurements have been done by determining one or more of:

1. Biomass accumulation (discussed in Ad-Hoc Committee Report, this section)
    a. Plate counts
    b. Turbidity
    c. Direct counts
    d. Protein determination
    e. RNA determination
    f. DNA determination
    g. ATP generation
    h. Dry weights

2. Utilization of reactants
   a. Substrate disappearance--important, necessary but preliminary only
   b. O_2 consumption--valuable tool but limitations (same as CO_2 below), especially stimulation of uptake due to uncoupling
   c. Organic carbon disappearance--preliminary screening tool only

3. Appearance of products of reactions
   a. CO_2--simple, inexpensive test used for preliminary screening of water-soluble compounds. Problems of stimulation of endogenous respiration and co-oxidation of non-substrate carbon.
   b. H_2S } used for anaerobic biodegradation evaluation, but limitations
   c. CH_4 } were not discussed
   d. NH_3, etc.
   e. Intermediate organics

4. Physical-chemical changes in medium (only qualitative measurements, to date)
   a. Surface tension--tool for slightly water-soluble compounds but not evaluated
   b. Color development or disappearance
   c. Viscosity changes
   d. Odor, appearance, etc.

The methods used to develop a value for the above are varied and many, and include the use of:

1. Respirometry (Warburg, Gilson, O_2 probe, etc.)
2. Radiorespirometry
3. Bunch and Chambers modification of RDA--used extensively by industry
4. Shake flask--both soluble and relatively unsoluble compounds
5. Activated sludge
6. Mineral slots and carbon source (+) or (-) growth factors

The inoculum may be:

1. Pure culture of a standard organism
2. Pure culture of a wild strain
3. Mixed culture of known organisms
4. Mixed natural population of unknown kinds and numbers of organisms.

Screening Protocols (Tier 1)

A. Activated Sludge Systems

The task force identified laboratory batch and continuous activated sludge systems as the best screening tools for the initial examination of water-soluble, non-adsorbing compounds for degradability. Examples of these systems were described by Gledhill in this workshop. Such systems have been used for many years and probably represent the most reproducible and economical method for initial screening for biodegradation. It was concluded that activated sludge from municipal plants probably represents the best compromise for a "standard" inoculum and would be readily available to most laboratories.

The use of dried, frozen and lyophilized sludge was considered for use as a "standardized" inoculum but the lack of experience in their use precluded further consideration. A common method of detecting utilization of the test compound during the initial stages is the measurement of total carbon disappearance. Specific analysis for substrate depletion in the supernatant liquid would further confirm biodegradation or the lack thereof.

It was concluded that operating an activated sludge test system past 15 weeks would not serve any useful purpose.

B. River Die-Away Test

This test, which has been used extensively to study degradation of surfactants, does not appear to have the utility of the activated sludge systems. The major drawbacks would appear to be non-uniformity and small size of inoculum. This type of test or modification for the marine environment could be recommended as a follow-up for the activated sludge system. Substrate concentrations are usually in the ppm range and the system therefore requires a very good analytical method for determining substrate disappearance and intermediate or end product formation. When available, C-14 labelled compounds can be used very effectively as shown by Lee in this workshop.

C. BOD Test--Not recommended as a primary screening tool.

D. Research Needs at the Initial Testing Level (Tier 1)

The task force believes there is an urgent need for research in the following areas:

1. Identification of a set of reference compounds for standardizing screening tests.
2. Identification of a "standard" inoculum to use in marine studies.
3. Development of standardized screening tests for insoluble compounds.
4. Development of an anaerobic screening test.

The amount of effort to be expended in the above would appear to be minimal and relatively short term.

Advanced Protocols (Tier 2)

A. Microcosms

It would appear from the work presented at this workshop by Pritchard et al. and Eggert et al. that the use of microcosms can be a useful tool for predicting the fate of chemical compounds in actual aquatic systems, such as lakes, rivers or estuaries. These systems have yielded reproducible results and seem to approximate the real environment.

In summary, the techniques for determining biodegradability of most compounds are available almost immediately. Very little, if any, new technology is needed. The most effort needs to be placed in standardizing tests so that results will be reproducible from laboratory to laboratory.

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TASK GROUP VI: PERSISTENCE AND EXTRAPOLATION

J. M. Tiedje, Chairperson

This task group dealt with the components affecting persistence, the definition of persistence, and valid extrapolations of persistence. Because persistence cannot be defined for regulatory purposes without consideration of the method used, features of test systems were also discussed and their principles determined.

I. Components of Persistence

A major point of consensus is that persistence is strongly influenced by environment and thus can only be discussed with reference to a particular natural environment or test environment. Components identified as affecting persistence which must be recognized in any persistence testing or definition are as follows:

1. Environment (nutrients, oxygen, pH, temperature, salinity, and water activity in the case of soils). When the testing method is the issue, additional environmental influences such as time since sampling, handling of subsample, method of addition of test compound, can influence the native state, and thus potentially the test result.

2. Concentration of Test Compound. We feel that it is undesirable to use a single test concentration. For determining test concentrations the following features must be considered: (a) concentration relative to K_t for uptake, since for concentrations far below K_t little uptake and thus metabolism would occur; (b) the toxicity of the chemical; and (c) its water solubility. The task force recommends that three concentrations be used. These should extend beyond the range of the expected concentrations in the habitat of concern.

3. Composition of Test Compound. Persistence of a chemical can be influenced by other associated chemicals such that degradation may either be stimulated or retarded. Chemicals can be tested as pure chemical, as the commercial grade, as the formulation, and with expected chemicals from other sources in the receiving environment. The task group recommends that the focus initially be on the pure chemical and commercial grade material, with judgment used to determine whether it is appropriate to test the formulation or the associated suite of chemicals expected in the receiving environment. There should be some flexibility allowed the company to test in a manner that is most representative of the use and expected fate for these latter two situations.

4. Other Physical-Chemical Factors. The task group feels that the other physical-chemical properties of the test molecule that affect its form and availability in environmental systems must be considered in any definition and testing of persistence. These include chemical and photo-chemical degradation, sorption, and partitioning.

The above plus the following must be part of any persistence definition.

5. Nonpersistence requires a *chemical change* of the test molecule. Transport out of one environment is meant to be excluded since the primary compound is still present but in another environment.

6. Time

7. Quantity Degraded. A quantitative definition of persistence must have these latter two components identified. It was not possible to arrive at specific values. However, it is recommended that in persistence testing, the disappearance of a compound be followed over two half-lives or for one year, whichever comes first.

8. Metabolites. These are to be included as a component of persistence when considering the parent molecule. There was sentiment for considering metabolites as new compounds when dealing with regulatory procedures but it was felt too risky to separate metabolites from the parent compound if the parent compound were termed nonpersistent when its metabolites were persistent.

9. Extent of Degradation. Acceptable intermediates are considered to be CO_2 and (controversial) irreversibly bound residues, the most common example being that of the metabolite covalently bound to humus. Standard intermediary metabolites are also acceptable products, however, they generally need not be formally considered since they should be rapidly metabolized to CO_2 and biomass.

II. Definitions of Persistence

Since persistence definitions are used for several purposes, our suggested definitions are specific for each purpose.

Dictionary definition--"Persistence is the tendency of a molecule to resist alteration by natural processes."

Scientific definition--"A persistent compound is a chemical and its metabolites for which most of the material remains in the receiving habitat after many months." The task group did not put quantitative values on the time/quantity-degraded component because persistence is really a continuum, and because there are no data to support any cutoff value; it is more a "feeling" based on experience with previous chemicals like DDT, PCBs, etc.

Regulatory definition--Here persistence needs a more quantitative definition. It was felt that this must be based on the test method. Thus, the basic principles of the method and their meaning follow.

III. Test Principles Important to Persistence and Extrapolation

Test 1--Principles: Complete mineralization, oxidative, short-term, low biomass/substrate ratio, and no specific analytical method required. The inoculum must be representative of the biological diversity expected in the likely receiving habitats. This test will identify those compounds which can serve as a carbon source for growth of microorganisms. The test will yield a yes/no result which can generally be extrapolated to nature, to several concentrations, and to most environments, though not from aerobic to anaerobic habitats.

Test 2--Test for "cometabolism." Principles: High biomass, substrate ratio, great genetic diversity of biomass, added energy sources, specific chemical method for compound required. It is felt that a pure culture and a synthetic sludge have too low a metabolic diversity to be useful inocula. A positive result indicates existence of biodegradation enzymes but extrapolation to degradation in nature would be risky. If the test conditions are reasonably similar to the receiving habitat such as may occur for a semi-continuous sludge, then this test has more merit. However, if they are very dissimilar, the value of this type of test is questioned.

Test 3--Test in subsample of receiving habitat (microcosm). Principles: Habitat samples must mimic the native state, concentration of test chemical should extend beyond the expected extremes in nature. ^{14}C-labelled materials and mass balances should be used if possible. It was agreed that rates obtained could not be extrapolated accurately to nature because of the diversity of habitats found naturally. However, relative biodegradability can be ascertained by comparison with standard compounds of known behavior. Since extrapolations will be made, the key environmental parameters affecting degradation should be identified and monitored in the test habitat and compared with those in the receiving habitat. To facilitate this extrapolation, simple and validated models can be used.

Since the central goal of this workshop and of the public in establishing new legislation is to protect the environment from serious toxicants, the biology and environment of any test must reflect what is likely to occur in nature. Limited biological diversity in test systems such as pure cultures, synthetic sludges, and other simple mixed cultures is inappropriate. We also feel excessive emphasis has been placed on standardization and uniformity in the workshop discussions. This is obviously expedient to both the regulatory agency and industry but it may sacrifice the basic intent of the law--to protect the environment from serious toxicants. Use of selected chemicals seems adequate as the element of standardization. Attempting to achieve a standard microbiological inoculum is an impossible task.

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DISCUSSION

SUMMARY SESSION

RAYMOND TASK GROUP

GRADY: I'm an engineer, a biochemical engineer, so I feel that I have the background for my statement. I have to take very strong issue with your statement that deactivated sludge would serve as an adequate inoculum. It seemed ironic to me that this group would argue over microcosm studies while not recognizing the vast differences in populations from activated sludge systems. That strikes me as incongruous; it flabbergasts me, quite frankly. By changing the operational conditions on an activated sludge system receiving a constant influent, I can change, totally, the species of organisms found in it. Consequently, to say that activated sludge would serve as a standard inoculum for doing biodegradation studies is totally fallacious. We have to have more than that. It upsets me very much that you would come up with that as a recommendation.

CLESCERI: I agree completely with Dr. Grady. I really was flabbergasted as well. I am kind of sorry that I spent all my time in microcosms, because I would like to have participated in this discussion. I feel very strongly that you're talking about a highly selected type of microflora in the activated sludge process. I understand the utility, but what's important is the recipient environment as far as the toxic organic compound is concerned.

LIU: During the last five years we have serviced 13 or 14 sewage treatment plants in southern Ontario. Every month we checked the bacterial populations and types in soils and in activated sludge. We found there is very little fluctuation, because most sewage treatment plants, if operated properly, will be the same. All sewage pipes consistently receive an inoculum of soil. We worked five years and we do not find differences (in terms of bacterial populations) from soils, to industrial effluents, to residential effluents. They're all the same. We have reliable data to use as back-up.

RODGERS: In the microcosms group, we discussed the idea of standardization. As far as a list of standardized chemicals to be shared by labs, we agreed with your statements on this basis. But for a standard inoculum, we felt it was much more important to consider the ecosystem or the receiving environment. For a number of organics, this would be ubiquitous. But as pointed out by our task group, there are many organics for which you can identify the area of application. We would feel much more comfortable using an inoculum that was representative of the recipient area.

DiGERONIMO: I have to defend the task group's decision to use sewage as a standard inoculum. Although populations may change in sewage, and granted you can alter the rate of degradation based on the flora present, we've found using chlorobenzoate as a model substrate that the potential was there. You do have to adapt or acclimate sewage to increase the rate of degradation, but I think the potential would be there. To say it's a ridiculous approach, I would disagree.

GRADY: You said the word "ridiculous," I said I was flabbergasted. Granted, the potential would be there, but if you take *any* activated sludge system and say the same potential is there, I take issue with that. There is going to be a limitation on the organisms which are present within that system that is governed kinetically by the interaction of these species. There is competition, and all sorts of ecological interactions, obviously. The way the plant is operated, the flow configuration, the flow rate, all have been shown in ecological studies to change the mix of the flora and fauna. My point is, if you say there is no potential for a degradation based on data from an activated sludge inoculum, I think you would be selling the system short, because inocula from other places could well show that potential. If you got a positive result, fine, but a negative result really doesn't tell you much. That's my point.

BETTINGER: The fact is that ecosystems in different parts of the country will be different, just as your sludges will be different. As Dr. Raymond said, we have to come to some kind of decision as to what we are going to screen. We have focused on planned application of compounds to the environment, but I think another interest of the Office of Toxic Substances in TOSCA is accidental introduction. You can't really screen for accidental introductions, transportation accidents or something like that. What are you going to decide on? You just can't have ten different soils or ten different areas to screen. I don't think that's economically feasible.

SHARP: I hope this does not transfer over into the regulations for pesticides.

RAYMOND: Well, let me say I'm really pleased to get this response. This becomes a rather dull meeting if we don't have a difference of opinion. I think we all missed something here. We have to put on our blinders. We can't argue about the 10% of things that don't fit into our patterns. We have to take chances. We discussed this at length in the task group. I am sorry that all of you were not there, because I can see we could have had some different inputs. You have to decide where your best chance of getting a uniform inoculum is, from anywhere across the country. A person could go out and obtain an inoculum from an activated sludge plant in almost every community. Yes, I know they differ from day to day, from month to month; I have operated them. So our task group was not totally ignorant of the chances of having spurious results from upsets in an activated sludge plant. Certainly, you can change the flora in a plant. But let's consider where in the United States or anywhere you would find the possibility of having almost every microbial species coming into one place. We argued about soil as an inoculum. This is not very uniform across the country. It is for some things like hydrocarbon oxidizers; you can find them almost universally. But you have to have some basis as a source of inoculum. I'm sure that this group would accept minority reports if someone would like to introduce one. I think I've said enough.

TABACK: Since you did mention soil as a possible inoculum in addition to activated sludge, what about a suggestion of mixing soil inocula with the activated sludge to make a standard inoculum? This would be sort of bridging the gap between some of the differences of opinion.

RAYMOND: That was brought up and, in fact, it was pointed out that there are some standardized soils brought into the National Bureau of Standards from around the country for other purposes. Of course, they don't take into account the biological makeup of these soils. That would have to be established. There is no mechanism, at the present time, to get uniform soils into the hands of most laboratories.

BERGMAN: In the microcosm group we discussed the same problem. We decided among other things, that as a first-tier test, which you're describing for the sludge, you need a conservative test. An environmental sample or inoculum would be more conservative. We also discussed the possibility of using, at the first two sets of tiers, a range of inocula from several sources, including a conservative and a more diverse bacterial inoculum if it's available.

BELLY: I think a number of us are not arguing with the facts that flora from sludge treatment plants vary. The question is, how constant is the degradation potential of the sludge? I think that sludge by far is the most reproducible as far as degradation potential is concerned. It does represent a mixed population for these studies. I think the flora may vary, but the question is, does the degradation potential vary? Maybe there should be more work in that area.

TIEDJE TASK GROUP

RAYMOND: Jim, I can't pass up the opportunity to take issue with you. It may appear there is an agreement here between industry and government, but I think we in industry are faced with practical realities. We have to test those compounds tomorrow, at least that's my opinion.

TIEDJE: I recognize that. My point is, uniformity and standardization, is it all that important? We have already standardization with use of selected reference compounds. Do we also need to standardize the biology in the test system?

BOLLAG: I don't see the possibility of a general consensus for methodology from a scientific point of view. EPA has to regulate industry, and scientists should participate in defining these regulations. Scientists can contribute in developing certain methods and participate in this kind of definition and criticism, but EPA has to prepare the suggestions for the regulation of industry independently.

RAYMOND: I have heard this mentioned at numerous meetings that I have attended both in societies and workshops like this. For some reason we in industry do not seem to be as scientific as we could be. We are concerned about whether something persists in the environment. We have to be; not only is it an economic problem, it is also a moral and social one. After all, we're not too different from you, as far as our needs and desires. We have to live in the same environment as well.

BOLLAG: One should accumulate all the available data on xenobiotics to foresee what their fates should be. At this conference I have a strong feeling that more than half of the data are not available for the general public. There are the scientific publications, but much more has been done in industry which is not disclosed. I think there is a certain moral issue which we should consider. I agree it is not a different world for industrial people and the non-industrial people, but one should try to change a certain secrecy so that more knowledge is available to everybody.

FLOODGATE: Just one comment on the use of lengths of time for defining the systems. We must remember that things can move out of the environment into another one during a period of time. For example, the residence time in the Irish Sea is about a year. In other words, anything that's put into the Irish Sea will be washed out in about a year if it is not sorbed to sediments. Whereas for another ocean, like the Mediterranean, residence time is 40 years. So you do have somewhat of a difference to consider where you want to give a particular time to define persistence.

TIEDJE: I agree. That's one of the reasons why we could not agree on a specific length of time as part of the definition of persistence.

SHARP: I think we're getting a little mixed up in terms of technical or scientific matters in the political process. What we're doing here is, hopefully, establishing a very solid technical basis upon which to make political decisions. Industry is, by and large, trying to do as scientific a job as possible. Each test we run, we attempt to have self-validating in terms of controls or positive controls. So I disagree a little bit with Dr. Raymond on that score. We are trying to be scientific, and we are trying to mold this technical information to satisfy and respond to the political process.

DAGLEY: Thank you very much. Any other comments?

SUMMARY OF THE CONFERENCE

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This conference has ranged over so many different disciplines which contribute to an understanding of our environment that an adequate summary by anybody is well nigh impossible. In consequence, the task was assigned to me on the basis of age. As Matthew Arnold showed, in a poem of rare and refreshing candor, the benefits conferred by growing old are very few, and are overestimated. The only advantage of age is that sensible people ought to acquire a useful perspective over a long span of time. This is not the same as wisdom: a bore at twenty usually matures into a crashing bore at seventy.

My perspective goes back over 40 years to the time when my first research papers were published in the Journal of the Chemical Society. In one of these papers we showed that we could establish exact quantitative relationships between amounts of carbon and nitrogen supplied to cultures in compounds serving as growth sources, and relate them to the sizes of the bacterial crops they supported. Since data of this type are now used routinely in continuous culture work, it may come as a surprise to learn that in 1938 people were skeptical about the handling of growth data in a quantitative fashion. At that time, as the chemist N. V. Sidgwick used to claim, bacteriologists might well have said their prayers to pH; but that was about all they were ready to take from physical chemists and mathematicisns. Cultures stopped growing, they believed, because all the biological space had been used up. This concept, like 18th century phlogiston, did not readily lend itself to mathematical manipulation. However, we are in no position to look down our noses at our scientific forebears: they had a clearer view of phlogiston than many of us have of biodegradability. I was reminded of the birth about four decades ago of quantitative bacteriology by hearing of Dr. Floodgate's much more difficult accomplishment: that of relating marine oil concentrations to nitrogen demand, although in this case the linking parameter of biomass is absent. In other words, the fate of nitrate consumed, as oil is removed, remains something of a mystery since it cannot be linked directly to nitrogenous cell constituents.

The contrast between oil spills and chemistats highlights the differences between the nature of the problems of the EPA and those of academic science. These differences relate to the nature of the questions that are raised. Science progresses by asking sensible questions that arise from reflecting upon an established body of knowledge. The questions for the EPA do not--they are prompted by urgent problems that are thrust upon society. Nevertheless, the best answers will be those based upon the broadest body of knowledge. They will not necessarily be the simplest answers; moreover, a bad answer may be worse than no answer at all. Let me illustrate with an analogy, and suppose that an EPA administrator is taken to the hospital to have his appendix removed. The policy of that particular hospital is dictated by economy and common sense. The operation must be the simplest possible, using a surgical knife costing no more than $5.75. However, I myself would prefer the procedure to be based upon scientific criteria, including the best anatomical knowledge available. Otherwise, our administrator might regain consciousness to discover that other, less dispensable, organs had been removed on the grounds that they were easier to get at. When a system is complicated, only a genius is able to ask simple questions that are not naive, or develop simple procedures of investigation that avoid artifacts. One complication arises from the fact that the questions themselves alter with time. Dr. Lichtenstein provided excellent examples to which I shall refer.

A dozen years ago, it appeared that there were two questions of paramount importance to answer. These were: (1) What structural features of a pesticide determine its binding to soils? and (2) Are you being misled into thinking that a particular pesticide is biodegradable, when in fact it has simply been swept under the environmental carpet? As Dr. Lichtenstein showed, the first question may change to another: What compound is being bound? For the chemical structure of the pesticide originally applied may have been modified. If it contained a nitro group, then strong binding may not have occurred until bacterial reduction provided an amino group. And by this time the second problem may have divided into several, as may be illustrated by asking a third question: To what part of the underside of the carpet is the modified pesticide now sticking? Both Dr. Lichtenstein and Dr. Pritchard believe that it sticks to detritus, but they are unable to reassure us that it is not only out of sight but also out of mind, because it appears that although man cannot get it off the carpet, worms can. But have the worms simply moved it to another place under the carpet? This is not a good question, since we do not know the precise meaning of "it" until we know all that the worms have done.

Dr. Bollag also provided interesting examples of questions that altered with time. He began with: How do microbes degrade carbaryl? The question soon became: How is the much more toxic compound 1-naphthol (derived from carbaryl) degraded? But this turned out not to be a good question. Behind it lies the assumption that fungi are in the business of degrading 1-naphthol; and indeed, some of them might be. But others, including those of Dr. Bollag, find it more interesting to synthesize polymers from 1-naphthol, and they only degraded a fraction of this substrate to completion. Moreover, some of the polymeric material became bound to soil components and stayed in that condition. It is probable that the fungi in these experiments used the same enzymes for polymerizing as those used for making secondary metabolites. But whatever they did, a knowledge of their metabolism, derived from academic studies, is an indispensable guide to understanding the rather surprising fates of these potential poisons. With such a background, one is less likely to ask Dr. Bollag simple (but as it happens, silly) questions such as: How is carbaryl degraded? or, Is carbaryl persistent? These questions have structural features that resemble those of a more famous question: When did you stop beating your wife?

Mention of amino groups and their connection with some aspects of persistence reminds us that such groups may pose other environmental problems. Herbicides classified as phenylcarbamates, acylanilides and phenylureas can be hydrolyzed by microbial enzymes to give chloro-substituted anilines. Dr. Kaufman's group studied the further metabolism of 4-chloroaniline by the soil fungus *Fusarium oxysporum* Schlecht. This organism activated the amino group, forming 4-chlorophenylhydroxylamine, so that noxious chemicals such as 4,4'-dichloroazobenzene and 4,4'-dichloroazoxybenzene could arise by chemical coupling. There have also been studies to show that the much used dinitroaniline herbicides can be reduced by soil microbes to give amino compounds.

Although the above mentioned transformations are brought about by living organisms, Dr. Plimmer did this workshop a service by stressing the importance of "straight" chemistry for environmental systems. Hydrolysis may be promoted by bringing a compound to a suitable pH, whether or not the change was due to microbial activity. Aldol fissions of carbon-carbon bonds are favored by high pH, particularly in the presence of certain metal ions. Reducing conditions and acidity have a profound influence upon the availability of metals in sediments, determining whether they will exist as free ions, as organic or inorganic complexes, as soluble or insoluble forms. In the time at his disposal, Dr. Plimmer could only touch upon the vast subject of photochemistry, but we must remember that numerous compounds undergo chemical changes when exposed to sunlight, and it is not satisfactory to ascribe these changes to microbes. Moreover, the world is full of surfaces, and physical chemists have written weighty tomes that bring out differences between homogeneous and heterogeneous reactions. Plimmer and Kaufman introduced a valuable note of caution in the session on Degradative Methodology, namely, watch your step when you sterilize. What soil enzymes--as distinct from microbes--will you remove? What new surfaces will you create? What free radicals will you generate if you sterilize by irradiation?

So far, I have not referred to yet another simple, common sense, and in my opinion quite naive, question which is asked repeatedly, namely: What features of chemical structure determine the biodegradability of a molecule? When you stick to purely chemical considerations you can give sensible answers to this question. If he knows the physical conditions and the chemical reagents for a proposed degradation, an organic chemist can make accurate predictions about its course. But microorganisms are not only subject to the laws of chemistry; what they can and cannot do is also determined by their evolutionary history. The conceptual framework, within which many environmental problems must be discussed, can only be constructed from an extensive knowledge of microbial catabolism. We must know something of the degradative capabilities of microbes in nature before we make sweeping generalizations about what they cannot do with man-made chemicals. Let me give three simple examples by way of illustration. All biochemical degradative processes occur by a series of reactions, each of which involves a small free energy change. No step is explosive; and as a consequence, there are usually many steps, liberating the total energy in small packets. It is therefore a matter of common sense that if compound A is to be converted, through the alphabet, to Z which represents CO_2, then the structures of B, C, and all the other compounds are also relevant to our considerations of biodegradability. It is not enough to concentrate solely upon the structure of A. As a second example, consider the well known effect of halogen substituents in blocking biodegradation. There are, indeed, good chemical reasons why a chlorine substituent might hinder enzymatic attack upon an aromatic compound. But it would be foolish to ascribe an anti-degradative property to chlorine atoms, because we have no evidence for this property of atoms. In the last decade a great many halogen-containing natural products have been identified that were previously unknown; this implies that there are many more microbes capable of eliminating halogens from organic chemicals than we had previously believed. Since most of these particular natural products are found in the sea, it is reasonable to suggest that this is where halogen-eliminating microbes are most likely to be found. As our third example, we might suggest that methyl substituents are an obstacle to degradation. If a carbon atom in the side chain of a detergent carries two of them, then this is indeed an observed fact which, incidentally, is readily explained with reference to the enzymology of β-oxidation. But it would also be foolish to ascribe a general anti-degradative property to methyl groups. A ring carbon of the natural product camphor bears *gem* methyls, and this compound is not only biodegradable: studies of the details of its catabolism by Dr. Gunsalus and his group have greatly enriched our knowledge of the mode of action of cytochrome P-450 in particular and of mono-oxygenases in general. In summary, I view with skepticism simple generalizations relating biodegradability to chemical structure. It is like using the simplifications of opinion polls to probe the complexities of the human psyche. A very rough guide is to try to relate the structure of the man-made compound to those of known metabolites. The greater the difficulty you have in doing this, the greater the difficulty microbes will have in degrading the compound. For further refinements in prediction, it is essential to know details of the probable catabolic pathway, so that degradative difficulties can be predicted as they arise along the route. However, it cannot, of course, be argued that because chemical linkages or groups in natural products can be identified in the symthetic compound under consideration, that this implies biodegradability. It is common knowledge that many man-made polymers are notoriously persistent, and yet they sometimes contain linkages found in nature. Thus nylon contains amide groups at regularly spaced intervals, and yet is resistant to proteolytic enzymes, including those that are extracellular.

In order to bring some of the themes of this conference together, I shall now make an adjustment in perspective. I shall go back even further than my student days at Oxford, with which I began this summary: instead of 40 years I shall go back 4 billion years to the origins of biochemicals. But first, I shall refer to a modern summary of metabolism, namely the metabolic charts which are often displayed upon the walls of offices and laboratories of departments of biochemistry. These charts summarize the central reactions, the cycles and sequences, found in most living cells. When reactions are examined in detail it appears that O_2 gas is effectively excluded from them. All the oxidations consist of removal of hydrogens. These are transferred to coenzymes, denoted as NAD, or FAD, and some of them may be used in synthetic reactions; but the rest find their way on to O_2, the very last reaction at the bottom of the map, thereby generating water. Let us skip by a vast amount of research on biochemical energetics and simply summarize with the statement that most of the energy liberated by degrading a biochemical is harnessed by the cell in reactions that occur just before the final rendevouz of hydrogen atoms (or, if you will, protons plus electrons) with O_2 gas. Oxygen was kept out of metabolic reactions until the very end. This is an overstatement that ignores a few vital reactions, but for most of the area covered by our wall chart we can say that direct reaction with O_2 is avoided. Anaerobic processes, analogous to those that operated when life emerged, have been preserved in aerobic organisms. You and I are essentially anaerobic in our metabolism except for the last few steps on the maps: the terminal (or final) respiratory chain, as these steps are called. We might note in passing that the ultimate source of the energy released in these catabolic sequences, if we trace it back, was solar radiation, trapped in photosynthesis. In the present atmosphere of the earth, containing O_2 gas, the stablest compound of carbon is CO_2. All organic compounds are unstable, in the thermodynamic sense, in this atmosphere, since they tend to be oxidized to CO_2 and H_2O. All organic compounds at present on our planet have been synthesized by a living organism using energy, whether that organism was a plant or an organic chemist.

What is this fortunate property of O_2 gas due to? This sluggishness under physiological conditions, so that it is kept away from the cell's inner biochemistry and yet is able to provide an energy gradient. Simply expressed: oxygen exists in a triplet ground state; it has two unpaired electron spins and the molecule is paramagnetic. Since carbon in organic compounds is in a singlet state, concerted reactions with oxygen are spin-forbidden; and in order to react, oxygen must be "activated" by absorbing radiation to attain a singlet state, for example, or by specifically combining with an enzyme. Haphazard activation would be catastrophic, because of the possibility that reactive O_2 might attack and destroy cell constituents. O_2 could enter our chart in the middle region and devastate metabolism. As it is well known, besides singlet O_2, there are other reactive forms. One is the free radical O_2^-, that is, oxygen having one extra electron. This is the superoxide anion. It is formed in traces, as a side product, by several of our own enzymes whose normal function is to do something else. There exists, in all aerobes, an enzyme, superoxide dismutase, which catalyzes rapid removal of the radical before it can do any damage. It is a widely held view that organic chemists and other higher forms of life were able to evolve, taking full advantage of aerobic conditions, because like all aerobes they had this particular protective enzyme. The reason why strictly anaerobic bacteria are condemned to lurk in the mud, and are soon killed in contact with O_2, is that they do not elaborate the dismutase. They are conservatives that cling to the anaerobic past. They have never been able to tolerate radicals, such as O_2^-.

However, although it is extremely hazardous for living matter to activate O_2, it became necessary to do it--*on purpose* and *not accidentally*. The reason for this was that plants were producing great quantities of biochemically inert material; some of it served obvious functions, such as that of lignin which was essential for structural rigidity, while many other natural products were synthesized for purposes that remain obscure to this day. It is fortunate that while green plant photosynthesis was giving rise to these rather inert natural products, it was also furnishing atmospheric O_2. For the rapid degradation of many of these compounds, leading to the rapid release of CO_2 to be used again in photosynthesis, can only be initiated by incorporating oxygen atoms into their chemical structures. This is the biggest single factor which determines the astonishing degradative versatility of aerobic bacteria and it is this versatility that we rely upon to cleanse our environment of waste and pollutants. Aerobic bacteria use most of the biochemical sequences and cycles that occupy a central position in metabolic maps and are generally found in other living forms. But in addition they possess the unique biochemical asset of being able to catalyze the safe and controlled oxidation of numerous natural products using molecular oxygen directly, incorporating it into molecules. In this way they start going reaction sequences that enter the central pathways of metabolism--such as the Krebs cycle or the fatty acid "spiral." Thus, an alkane remains biochemically inert until a terminal carbon has been oxidized. The fatty acid formed by inserting oxygen may then enter a main channel of metabolism, namely, β-oxidation. A saturated ring-structure is also inert until a hydroxyl group is inserted. If this occurs at an appropriate point in the molecule, the possibility then arises that further oxidation may break the ring, providing fragments that can be degraded. The unsubstituted benzene nucleus is an inert resonance structure, but when suitably substituted with two hydroxyl groups, using oxygen directly, enzymic ring-fission may then take place. The enzymes used are dioxygenases. They activate O_2, and this is risky; but for ring cleavage, or hydrocarbon oxidation, it is necessary to do it.

Degradative reactions are just as important as synthesis. Half a carbon cycle is no more use than half the wheel of a bicycle. And to carry out the necessary molecular decomposition, the mass (not the number) of microbes on this planet far exceeds the mass of animals, let alone that of human beings. Without them, evolution could not have occurred since cycles such as those for carbon, nitrogen and other elements provide the condtions for evolution to occur. They operate in a stream of solar energy. They involve life, death and decomposition.

These general considerations form the background to Dr. Chapman's presentation. The "special" microbial enzymes used in aerobic degradative pathways he described for natural products are the oxygenases. By comparison, the range of these enzymes possessed by higher organisms is extremely restricted. After degradation has started, however, the *types* of reactions are those found generally in biochemistry: dehydrogenases, decarboxylases, hydratases, aldolases, and so on. Such enzymes are assembled in suites in a very elegant fashion, to provide smooth entry of material into the Krebs cycle. Quite often, industrial products such as cresols and xylenols can be accommodated by the enzymes used for catabolism of natural aromatics, so that these compounds are also biodegradable when they are present at low, non-inhibitory concentrations. It was also pointed out in this conference that microorganisms capable of degrading polynuclear aromatic hydrocarbons (PAH) are quite readily isolated, probably because these compounds have been present with us ever since forest fires began, and in consequence can be regarded as natural products and can serve as perfectly good microbial nutrients. Dr. Gibson and his group, who have done so much to elucidate this area of microbial catabolism, reminded us that PAH also arise from motor vehicle emissions, coal-liquefaction and gasification processes, cigarette smoke and industrial wastes. Low concentrations of PAH find their way into marine ecosystems. It is a matter of common knowledge that the introduction of halogen atoms into the structures of PAH obstructs the action of biodegradative enzymes and renders these compounds persistent.

One of the basic principles of enzymology is that some enzymes are more specific than others toward their substrates. Accordingly, the analog of a natural substrate might be attacked by the first few enzymes of a degradative sequence, because their substrate specificities are broad enough to tolerate small modifications of structure. However, somewhere along the line a catabolite may arise that can no longer be accommodated by the next enzyme in the sequence. The pathway is then blocked and degradation ceases. If the enzymatic reactions have furnished neither nutrients nor energy up to this point, then this partial degradation will satisfy Dr. Alexander's definition of cometabolism. As he points out, there may be other biochemical conditions giving rise to this situation; but the crux of the matter is that the cometabolic chemical does not serve as a nutrient source, and its presence in a natural ecosystem gives no selective advantage to the organism acting upon it. Dr. Alexander did a service to the workshop by reminding us of this definition of cometabolism, since the term has not only been misapplied: it has been used in the research literature, and in at least one review of cometabolism, in precisely the opposite sense. On these occasions it has been used to refer to those systems, so common in natural environments, where a chemical does not serve as a source of energy by itself but *will* do so when other compounds are present; or when a second microbial species profits by the abortive degradative start made by the first (which, in the end, may also benefit by assimilating excreted metabolites otherwise unavailable). There is more at stake here than semantics or definitions. It is a matter of stimulating, or discouraging, painstaking scientific investigation. When a compound disappears from a system only when changes in its chemical or microbiological environment are brought about, it may be necessary to undertake a fairly complex analysis before we can understand precisely what has happened. To say that its disappearance is due to the "phenomenon of cometabolism" simply avoids a proper analysis; and in view of the complications so often involved, this is about as helpful as saying that Beethoven's Fifth Symphony is due to the phenomenon of sound. This workshop has been fruitful in providing instructive examples of biochemical and microbiological interactions. I shall mention some of them, and a few others besides. None falls within the strict definition of cometabolism.

(1) *E. coli* typically does not permit the entry of citrate into the cell unless a rather complex mixture of nutrients is also added to the culture. Citrate is then metabolized. If you wish to give a special name to the phenomenon, which I do not recommend, you could call it copermeation.

(2) The white-rot fungi *Phanerochaete chrysosporium* and *C. versicolor* do not grow with, or decompose, lignin until a growth substrate such as cellulose or glucose is added. Decomposition of lignin ceases when the carbohydrate is used up. Some bacteria can decompose and grow upon lignin in the absence of carbohydrate, however.

(3) In this age of science fiction and overstatement, one has heard the word "superbug." With this background there is a temptation to think that a microbe capable of degrading a vast range of exotic structures is also capable of performing any chemical feat required. In fact, it may not be able to synthesize a particular coenzyme it needs: its degradative capacity may be remarkable, but not its ability to synthesize. It may need another and less

spectacular species as a growth companion to supply the required cofactor while it (the first organism) generates readily assimilated growth substrates. Dr. Slater described how a *Bacillus* that required the growth factor nicotinic acid excreted biotin and grew in the presence of a pseudomonad that required biotin and excreted nicotinic acid.

(4) In further discussion of the scope and limitations of continuous-flow culture techniques for studying interacting associations of microorganisms, Dr. Slater also described how one microbial species used H_2S photosynthetically while a second used the sulfate formed, converting it back again into H_2S. The last named compound was toxic to both species, but both flourished because the microbial association enabled the concentration to remain at a low, non-toxic level. It would be possible, but not particularly profitable, to call this state of affairs codetoxication.

(5) The chemostat (continuous-flow culture) has opened up new areas for environmental studies by showing that new enzymatic activities can arise in stable associations of microorganisms. Dr. Slater mentioned a seven-membered microbial community that remained stable over thousands of hours in a chemostat limited by Dalapon (2,2'-dichloropropionic acid) as carbon source. During this period, four microbial species that lacked the ability to degrade Dalapon were able to exist upon excreted metabolites and cell lysis products arising from those members of the community that were metabolizing the herbicide. However, after 2,900 hours of continuous growth, a species of *Pseudomonas* belonging to the groups of "hangers on" acquired an active dehalogenase and joined in the work of the three primary utilizers of the carbon source. A similar situation existed for a stable mixed-species community supported by orcinol, when another passive pseudomonad eventually became capable of degrading the primary source of carbon. It is clear that the chemostat provides secondary utilizers (the "hangers on") with a continuous supply of nutrients arising from primary utilizers, while exposing them at the same time to the compound they cannot degrade. This is an excellent way to select competent mutants without the use of mutagens. While there are many questions to be answered, such as those relating to the stability of these microbial associations, one evident advantage is the wider genetic base that is provided by a stable mixed culture, compared with a monoculture. Many exciting studies are being undertaken currently to show how degradative capabilities can be transferred throughout the microbial world by the agency of plasmids. Work with the chemostat might indicate, one day, how the plasmids came to be there in the first place.

As I mentioned earlier, aerobic organisms make full use of the energy gradient that exists between photosynthesized biochemicals and CO_2. With such a simple end-product, and with energy available in abundance, the evolution of diverse individual living forms was favored. In the absence of O_2, life is harder. Degradation often proceeds in limited stages, each brought about by particular anaerobic species to yield an organic compound that will serve as a source of energy for the next set of organisms. In this way the "food webs," described by Dr. Poole, are set up under anoxic environmental conditions. In each segment of the web, the energy released is much less than it would be under aerobic conditions with CO_2 and H_2O as end-products. Moreover, for man and some microbes, H_2S is a far less acceptable end-product of metabolism than

H_2O. This compound can arise through the activities of a food web sustained by cellulose present in effluents from paper manufacture. Stages in anaerobic decomposition encompass hydrolysis of cellulose to oligosaccharides and their degradation to lactate and acetate that serve as carbon sources for microbes reducing sulfate to H_2S. If this H_2S accumulates, it will inhibit or poison microbes and higher organisms in earlier segments of the food web; however, the photosynthetic green and sulfur bacteria can remove sulfide, using it as a source of electrons, but at the expense of replacing H_2S with granules of elemental sulfur. One further example of the microbial complexities of anaerobic environments may be cited from the communication of Drs. Healy and Young. Investigations of aerobic aromatic catabolism such as mine have been simplified by the fact that any good undergraduate junior can isolate from soil a pure culture of bacteria, capable of growing aerobically with benzoic acid as carbon source, within the space of a week. But the converion of the benzene ring into CO_2 and methane anaerobically is harder work, and it takes a consortium of organisms to do this. The benzene ring has to be reduced and opened before the business of fermentation proper can begin, and it is not surprising that a microbial division of labor is called for.

During the course of this conference, ample recognition was given to the fact that aquatic environments may encompass regions of anoxia, as mentioned, and also bottom sediments as well as surface microfilms. Each has its associated biochemistry and microbiology, providing conditions that influence fates of pollutants in different ways. And yet these are usually regions, or components, of one complete system; and Dr. Clesceri showed that this complexity can be modelled using a computer, so that responses to perturbations can be simulated. It appears, incidentally, that those organisms that are least upset by environmental changes, and are most resilient in their responses, are the heterotrophic bacteria. Dr. Wright described how they become dormant when nutrients are depleted, and how they respond very rapidly when nutrients become available. Initially, these responses are independent of cell division.

As I observed at the beginning, it is very difficult if not impossible to summarize a conference of this breadth. I have not referred to many of the contributions that interested me; for had I done so, this would have been tantamount to promoting a series of satellite workshops. We have all benefitted from this conference: academic scientists as well as those whose difficult task it is to investigate, or give immediate advice on, urgent pollution problems. People like myself who work with pure microbial cultures grown at the expense of single carbon sources, preferably overnight, have been reminded that these conditions are far removed from those of natural microbial habitats. However, these approaches were adopted because it is wise to ask sensible questions of nature one at a time. To recognize complexities does not mean that we should abandon this attitude. We cannot afford to do so: we must ask more questions and design more adequate experiments. Attempts to deepen our understanding of the environment somtimes appear to proceed with frustrating slowness, but in the long run they will be more profitable than compiling extensive catalogs of undigested empirical data.

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APPENDIX

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WORKSHOP: MICROBIAL DEGRADATION OF POLLUTANTS IN MARINE ENVIRONMENTS
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